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# THE ELECTROPHORETIC MOBILITY OF HUMAN ERYTHROCYTES—WHOLE CELLS, GHOSTS, AND FRAGMENTS

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The results of some preliminary experiments, involving comparison of the electrophoretic mobility of the ghosts (stromata) of human erythrocytes with whole cells, and of the fragments of cells with whole cells and ghosts, are reported in this paper. Abramson (1) found that ghosts have the same mobility as whole cells in buffered isotonic suspension. This observation has been confirmed. However, we have observed marked differences when a foreign serum is present. Fragments of ruptured cells differ in mobility from the original cells, even in the absence of foreign material. These results suggest a new approach to the study of the composition and structure of the red cell wall, and a possible new method for clinical study of blood in disease.

A modified Northrop Kunitz or Abramson microelectrophoresis cell was used. The method has been adequately described by Moyer (2). Measurements on seven different suspensions of normal cells, in  $M/15$  phosphate buffer at pH 7.4, made on 5 different days, gave values of mobility in  $\mu/\text{sec}/\text{volt}/\text{cm}$  at  $25^{\circ}\text{C}$ , ranging from 1.25 to 1.31, with a mean value of 1.29. This compares with the standard value of 1.31 given by Abramson (1) and Moyer (2). Each result reported is based on a number of individual observations. The standard deviation for normal whole cells varied between 2.4 and 5.9 per cent.

## *Ghosts and Whole Cells*

Normal cells suspended in  $M/15$  phosphate buffer at pH 7.4 were hemolyzed by two methods involving no change of chemical composition of the medium, ultraviolet irradiation and dilution. The latter

method was used in most cases. Some of the cells resist hemolysis for hours so that simultaneous measurements on whole cells and ghosts may be made. Within the limits of experimental error, no difference was ever found between the mobilities of ghosts and whole cells in pure salt suspension. Neither was any difference found when 4 per cent human serum was added to the buffer solution.

Upon adding chicken serum to a diluted suspension of human red cells, the mobility of the ghosts decreases while that of the whole cells remains unchanged. The mean mobility of the ghosts decreases with time, and, after a few hours, many of them exhibit irregularities on the surface. These may be fragments from ruptured cells clinging to the surface or cell material resulting from extrusion. Whatever

TABLE I

*Effect of Chicken Serum on Human Red Cell Ghosts in M/30 Phosphate Buffer*

Serum	$v(\mu/\text{sec}/\text{volt}/\text{cm})$		Difference
	Whole cells	Ghosts	
<i>per cent</i>			<i>per cent</i>
2	1 57	1 47	6 4
6	1 57	1 33	15 3
8	1 47	1 10	25 2
10	1 51	1 00	33 8

the source, such irregular cells usually move more slowly than smooth cells. But, even before any rough cells appear, individual differences are much greater between ghosts than between whole cells. These facts place limitations on any effort to study quantitatively the variation of the effect with serum concentration. However, the nature of the mechanism is of such interest that such a study was undertaken.

A standard technique designed to avoid possible variations due to method of mixing, concentration of salt, and timing, was adopted. Whole cells were measured within 20 minutes after mixing, and ghosts between 30 and 90 minutes. The results are given in Table I. With this method, it was not possible to get significant results below 2 per cent serum, because of experimental error. The linear variation

with serum concentration appears to indicate a mechanism involving a chemical type change. However, nothing is known about the concentration of active material, and equilibrium is not attained. Therefore, a simple mechanism involving adsorption of a substance giving a lower over all charge density is not excluded.

Whole cells may be kept at room temperature in  $M/15$  phosphate buffer for only a relatively few hours without change of mobility. By reducing the temperature to  $6^{\circ}\text{C}$ , a suspension was kept for 7 days without change. With this method, the normal rate of change can be so reduced that any effect due to simple adsorption should be observable. But a 1 per cent foreign serum sample was kept for 11 days without appreciable decrease of mobility of whole cells and with no difference between ghosts and whole cells. It is, therefore, concluded that there is no direct effect on mobility of the cells from adsorption of foreign serum. At room temperature the active material in the foreign serum serves to catalyze a change which takes place even in its absence. Apparently, only ghosts are capable of reacting with the catalyst. Important information may be obtainable by testing known compounds for catalytic action.

Such a response to foreign serum suggests that abnormal substances resulting from disease may possibly be capable of catalyzing the reaction. A few samples of blood of patients were examined, and small effects were indicated in some cases, but the number of observations was not sufficient to allow definite conclusions.

### *Fragments and Whole Cells*

Some cells break up in  $M/30$  phosphate suspension to yield fragments of varying size. The behavior of fragments in pure salt suspension is similar to that of ghosts in foreign serum suspension—a marked decrease in mean mobility with time obtains and different fragments vary widely. The results from one experiment are summarized in Table II. It seems significant that the fragments have the same mobility as the ghosts immediately after breaking off. This indicates that the interior surfaces are similar in structure to the exterior, and perhaps identical. The relationship appears to be independent of hemolysis for fragments were produced mechanically



by grinding in a mortar in a M/15 suspension, with essentially the same result

At the outset, it was thought that adsorption of serum proteins by the fresh surfaces could account for the decreasing mobility of fragments. Serum-coated quartz particles were found to have a mean mobility of 0.81 in M/30 phosphate + 4 per cent serum suspension. With this in mind, a series of experiments was run with the normal amount of serum, with all serum washed out, and with excess serum. But the results were always similar.

The mechanism of this change is probably similar to that which accounts for the behavior of ghosts in foreign serum and of whole

TABLE II  
*Change of Mean Mobility of Fragments with Time*

	Time interval after diluting (min)			
	0-20	20-30	30-90	135-150
Mean mobility (g)	1.57		1.46	
( $\mu$ /sec/volt/cm at 25°C) (f)	1.55	1.16	0.91	0.69

cells on long standing. A chemical change, with which the decreased mobility is associated, possibly simply a rearrangement, is normally proceeding slowly even in whole cells. Upon hemolysis, the cells are able to adsorb certain substances which catalyze the change. After disintegration of the cells, the change proceeds more rapidly.

#### SUMMARY

The electrophoretic mobility of human red cell ghosts decreases in the presence of chicken serum. The decrease is not directly due to the presence of adsorbed material but to a change which is catalyzed by the foreign substance. It is suggested that abnormal serum materials resulting from disease may serve as catalysts.

Fragments of broken cells have the same mobility as whole cells at first, then decrease even in pure salt suspension, while the whole cells remain essentially unchanged for hours.

The results suggest that the slow change of whole cells, the change

of ghosts in the presence of foreign serum, and the change of fragments are all manifestations of the same modification of structure or composition of the cell surface

We wish to express our appreciation of the help and encouragement given by Dr W R Whitney

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# INTENSITY DISCRIMINATION IN THE HUMAN EYE\*

## II THE RELATION BETWEEN $\Delta I/I$ AND INTENSITY FOR DIFFERENT PARTS OF THE SPECTRUM

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### I

#### *Purpose of This Research*

When  $I$  and  $I + \Delta I$  are two light intensities which can just barely be recognized as different, then the fraction  $\Delta I/I$  is considered the measure of intensity discrimination. The value of this fraction and its relation to the intensity  $I$  have been the subject of many researches, and these have established that as the intensity  $I$  increases, the fraction  $\Delta I/I$  decreases, tending toward a minimum value at the highest intensities. This is true not only for the human eye, but for all other organisms thus far studied, namely, *Drosophila*, the bee, and *Mysa* (for a summary, see Hecht, 1937a).

Besides the general relation of  $\Delta I/I$  to  $I$ , the human eye shows an additional phenomenon due to the duality of its retinal structure. There seem to be two relations of  $\Delta I/I$  to  $I$ , one at the lower intensities representing rod function, and the other at high intensities representing cone function. This rod-cone dichotomy is apparent in nearly all the published measurements from the earliest by Aubert (1865) to the most recent of Steinhardt (1936). In addition, Steinhardt showed that the double function appears only in measurements with central visual fields larger than  $2^\circ$ , while the single function appears with fields smaller than  $2^\circ$ , and this corresponds to the presence of rods and cones in the larger fields, and to the complete absence of rods in the smaller fields. Moreover, the extent of the low intensity rod section increases with the size of the field because of the increasing number of rods present in comparison with the number of cones.

\*The first paper in this series is by Steinhardt (1936)

The rods and the cones possess different sensibility distributions in the spectrum, and this has been used to separate the two systems in measurements of dark adaptation (Kohlrausch, 1931), intermittent stimulation (Hecht and Shlaer, 1936), visual acuity (Koenig, 1897, Hecht, 1937 *a*), and instantaneous threshold (Blanchard, 1918, Hecht, 1937 *b*) For intensity discrimination this would mean that in measurements made with extreme red light, the relationship between  $\Delta I/I$  and  $I$  would be a single high intensity function even when the field is large, while with other parts of the spectrum more and more of the low intensity section should appear as the wavelength of the light moves toward the blue The data of Koenig and Brodhun (compare Hecht, 1935) show precisely this for red, orange, yellow, and green lights However, their data (Koenig and Brodhun, 1888, 1889) with blue, violet, and even white lights show no break between the higher and the lower intensities, instead the points form a flat continuous function which corresponds neither to their own measurements with the other colors, nor with those of Blanchard, of Aubert, and of Steinhardt with white light We have therefore investigated the situation anew, making measurements not only with different parts of the spectrum, but with white light as well, in order to establish the relation definitively, and to confirm the identification of the two sections of the function

## II

### *Apparatus*

We started with the actual materials of Steinhardt's original apparatus *B*, and rebuilt them into a new instrument<sup>1</sup> Steinhardt used two light sources, one for  $I$ , and the other for  $\Delta I$  This introduces obvious difficulties in control Our apparatus therefore uses only one light source A diagram of the optical system is shown in Fig 1

The light source  $S$ , which is a glowing ball of tungsten in an arc furnished by a Punktlcht, is at the principal focus of the two lenses  $L$  and  $L'$  These two lenses then start completely symmetrical optical paths The light emerging from the lenses  $L'$  and  $L$  is reflected by the prisms  $P_1$  and  $P_2$  respectively, and is brought to a focus at  $S_1$  on a half-silvered mirror by means of the lens pair  $L_1$  and  $L_2$ , and the corresponding  $L_1'$  and  $L_2'$  At  $S_1$  the two images of the source

<sup>1</sup>In doing this we had the help and advice of Dr Simon Shlaer, to whom we gratefully acknowledge our indebtedness

overlap. The reflected light of one and the transmitted light of the other then traverse a common path to the ocular,  $O$ , which focuses an image of  $S_1$  at  $S_2$  in the plane of the pupil of the eye. The size of the image  $S$  is about  $1 \times 1.5$  mm. This image becomes an artificial pupil, since its size remains constant at all intensities, and it is smaller than any pupil size achieved even by the most intense illumination.

What the eye  $E$  sees may be made out in terms of the dashed construction shown in the figure. A point on the lens  $L_1$ , which is at the focal distance of the

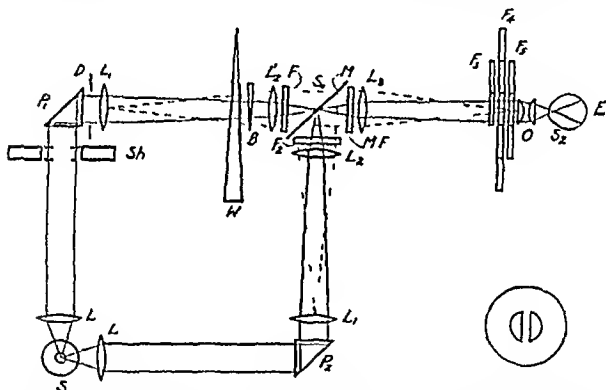


FIG. 1. Diagrammatic top view of the apparatus.  $S$  is the light source, and  $S_1$  and  $S_2$  are its two double superimposed images formed by the two trains of lenses  $L, L_1$  and  $L', L_1$ , and the ocular  $O$ .  $P_1$  and  $P_2$  are right angled prisms,  $Sh$  is a shutter,  $D$  a diaphragm,  $W$  is a neutral wedge and  $B$  its balancer,  $M$  is a half silvered mirror.  $F_1$  to  $F_5$  are neutral filters.  $MF$  is a monochromatic filter. The actual appearance of the field to the eye  $E$  is shown in the lower right corner.

lens  $L_2$  produces a parallel beam which is reflected or transmitted at the mirror and forms an image by means of the lens  $L_3$  in the front face of the ocular  $O$ . In other words, the eye, looking through the ocular, sees an image of either the lens  $L_1$  or the lens  $L_1'$  or of both superimposed and the two may be brought sharply into focus. The lens  $L_1$  furnishes the intensity  $I$  and  $L_1'$  the added intensity  $\Delta I$ . The appearance of the luminous surface of the lens  $L_1'$  may be varied by means of diaphragm  $D$  to form any chosen pattern. We have found it convenient to have the diaphragm represent two half circles subtending a visual angle of  $12^\circ$  and separated by an opaque bar  $3^\circ$  in width. The appearance of the field is shown in the inset.

where the larger circle represents the prevailing intensity  $I$ , and subtends an angle of about  $40^\circ$  at the eye. The stippled half circles represent the pattern of the intensity  $\Delta I$  superimposed upon this field of view.

In the path of the  $\Delta I$  beam are a shutter  $S$ , so that the duration of exposure of  $\Delta I$  may be controlled, and a neutral wedge and balancer  $W$  and  $B$ , having a range of 1-100 for the purpose of gradually varying the intensity of the  $\Delta I$  beam. In addition, the neutral filter  $F_1$ , which has a density of 1 may be inserted to decrease the intensity of the  $\Delta I$  beam by  $1/10$  when this is necessary. This is useful when the value of  $\Delta I/I$  is very small. On the other hand, when  $\Delta I/I$  is large, it may be necessary to insert the neutral filter  $F_2$  into the path of the  $I$  beam. Filter  $F_2$  also transmits  $1/10$ , and is useful as well in making a comparison of the absolute brightness between the  $I$  and  $\Delta I$  beams.

The combined  $I$  and  $\Delta I$  beams pass through the monochromatic filters,  $MF$ , which are the Wratten monochromatic series 70-76 plus the Corning 428 already used (cf. Hecht and Schlaer, 1936) for isolating different parts of the spectrum. In addition, both beams pass through a series of three neutral step filters,  $F_3$ ,  $F_4$ , and  $F_5$ . Filter  $F_3$  has two steps whose densities are respectively 0 and 4.  $F_4$  has five steps whose densities are respectively 0, 1, 2, 3, and 4. Filter  $F_5$  is in three steps whose densities are 0, 0.3, and 0.6. Thus, with the three filters in the position of 0 density the brightness is maximal. The brightness may then be reduced in steps of 0.3 log unit down to a total density of 8.6, which easily covers the whole range of intensities over which the measurements need to be made.

The lenses, prisms, and filters, as well as the wedge, mirror, lamp, etc. are properly set and housed in metal mounts so that no stray light is visible. However, in order to avoid the stray reflections from the lamp for reading the wedge and ammeter, the observer sits in a cubicle open at the back, into which projects the ocular arm of the apparatus.

All the filters were calibrated with a Martens polarization photometer, using the method described by Hecht, Schlaer, and Verrijp (1933). They were calibrated for each of the monochromatic filters separately, as well as for the white light. The neutral wedge and balancer were also calibrated with the Martens photometer for white light and for each of the monochromatic filters.

In order to compare the  $\Delta I$  and  $I$  beams, a diaphragm is inserted near  $L_1$  and near  $L_1'$ , these diaphragms cut off symmetrical halves of the respective fields. With filter  $F_2$  in position and filter  $F_1$  out, the wedge is moved until the two half fields match. Knowing the transmission of the  $F_2$  filter, and the brightness of the  $I$  beam, we secure the brightness of the  $\Delta I$  beam at that position of the wedge. From the calibration curve of the wedge, one can then compute the value of  $\Delta I/I$  for any position of the wedge and any combination of  $F_1$  and  $F_2$  filters.

The maximum brightness achieved with white light is about 1,000,000 photons. This was determined by making a binocular match with a semicircular field formed on an opal glass illuminated with a lamp whose distance was variable. The right eye looked at a half field in the ocular, while the left eye looked at the variable

comparison field through an artificial pupil. When the fields were matched, the outside field was measured with the Macbeth illuminometer.

### III

#### *Procedure*

The observer was dark adapted for 15 or 20 minutes before any measurements were made. He then started with the lowest illuminations and worked up to the highest. The observer sat properly shielded with his chin in a rest and his eye placed near the ocular, and looked centrally at the large field of intensity  $I$  for at least a minute until he was adapted to it. The wedge was then placed at such a position that an exposure of the  $\Delta I$  beam for  $1/25$  of a second by means of the shutter  $S_2$  produced an increase in brightness which was below the threshold. The wedge was then moved to increase the value of  $\Delta I$ , and an exposure made again. This was continued at 20 or 30 second intervals until the position of the wedge was found at which the eye could just clearly distinguish the pattern produced by the flash of the  $\Delta I$  beam. The wedge was reset below the threshold and the observation repeated. If the two readings were very near each other no third reading was made, otherwise, a third setting of the wedge was made and an average taken.

The intensity was then increased 0.3 log unit, and the whole procedure repeated. This was continued with increasing intensities until the complete function was established. Such a run took between 1½ to 2 hours. We made three runs each for five portions of the spectrum and for white light. Before each run the match point of the  $I$  and  $\Delta I$  beams was determined but this varied only slightly over a year.

### IV

#### *Measurements*

Measurements were made with our own eyes, J C P and M P served as subjects for the whole series of runs, while S H made the preliminary measurements as well as an occasional run which proved so similar to those of the other two observers that he did not complete the series. The data are given in Table I.

The measurements with white light are shown graphically in Fig 2, where the points for M P have been lowered 0.5 log unit for convenience. They are plotted as the logarithm of  $\Delta I/I$  against the logarithm of the intensity  $I$ , because only in this way can the nature of the results be realized in view of the enormous range of intensities covered and the rather large range of the fraction  $\Delta I/I$ . By plotting the data in logarithmic form the percentage error occupies the same





space at all intensities and all values of  $\Delta I/I$ . Moreover, the form assumed by the results is not changed by the actual numerical values of the fraction or by the units in which the intensity is measured.

The measurements with white light corroborate the work of Aubert, of Blanchard, and of Steinhardt in showing a sharp transition between the low intensity section and the high intensity section. It is hard to understand why the measurements of Koenig and Brodhun show

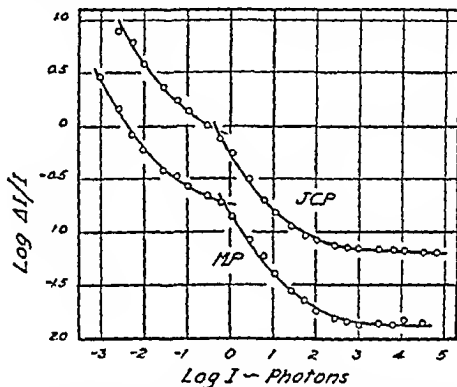


FIG 2 Intensity discrimination for white light where  $I$  is a field  $40^\circ$  in diameter, and  $\Delta I$  is  $12^\circ$  in diameter exposed for 0.04 sec. The points for M P have been lowered 0.5 log unit for convenience in keeping the two observers apart. Note the two sections in each set of data, note also that at high intensities  $\Delta I/I$  reaches a constant minimum. The curves are all from the equation  $\Delta I/I = c[1 + 1/(KI)^{1/2}]^2$  derived on theoretical grounds.

no break, especially since their data with orange, yellow, and green lights do show it. With us the break has always shown up under the appropriate conditions, though it may be added that when Steinhardt first found a break in his measurements, it was unexpected because of our reliance on the data of Koenig and Brodhun. When the observer is tired and his fixation and attention are poor, he occasionally gives results which, like those of Koenig and Brodhun, show no clear break. This may have been the case also with the data of Holway (1937).

Another significant point about the measurements in Fig 2 is that the value of  $\Delta I/I$  does not rise at high intensities, but reaches a minimum at which it remains no matter how high the brightness. This also contradicts the measurements of Koenig and Brodhun who reported a rise in  $\Delta I/I$  at high values of  $I$ . However, this aspect of the matter is understood, the rise does not occur when adequate adaptation is allowed and when an adequately large field surrounds the test field. The rise also fails to appear in intensity discrimination measurements with *Drosophila* (Hecht and Wald, 1934) and with the honey bee (Wolf, 1933).

The data for the five selected portions of the spectrum are shown graphically in Fig 3. The intensity scale on the abscissa is the same for all the colors, and is in Troland's photon units (Troland, 1916). We obtained these brightness values in two ways. One was by the heterochromic matching of the monochromatic filters among themselves and against white, while the other was by the superposition of the high intensity cone portions of the data for the different colors, which assumes that a given brightness produces a given value of  $\Delta I/I$  regardless of color. The differences between the two methods were so small that, knowing the errors of heterochromic photometry, we have actually used the superposition method in presenting the data. The scale on the ordinates applies only to yellow (575 m $\mu$ ), the orange (605 m $\mu$ ) and red (670 m $\mu$ ) measurements have been moved up by 0.5 and 1.0 log units respectively, while the green (535 m $\mu$ ) and blue (450 m $\mu$ ) measurements have been moved down 0.5 and 1.0 log units respectively. The data in Fig 3 are those of J. C. P. only because those of M. P. are essentially the same.

Judging by the measurements in Table I, it might seem that the lowest  $\Delta I/I$  values achieved differ for the different spectrum portions. This is an artifact due to the circumstance that the measurements were made over the course of many months at odd times and in no special order. During this time the observers varied to a certain extent, but what is more important, the shutter in the apparatus varied because the apparatus was demonstrated frequently and this involved resetting the shutter. When this long period variation became evident, we deliberately tested the minimum  $\Delta I/I$  at high intensities for the different colors at one sitting. The measurements

were made on the eye of J C P and the  $\Delta I/I$  values secured were 0.138, 0.125, 0.116, 0.114, 0.129 for blue, green, yellow, orange, and red respectively, thus gives a maximum variation of about 10 per cent from the mean, and is probably of no importance

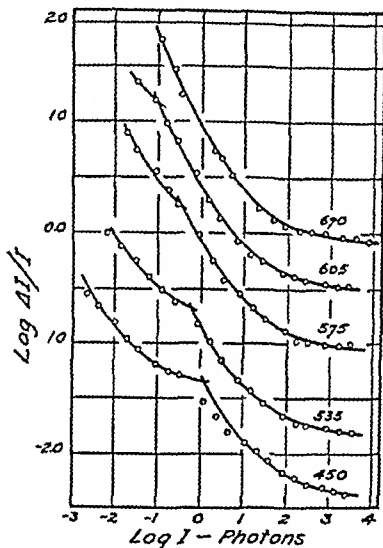


FIG 3 Intensity discrimination for the red, orange, yellow, and blue parts of the spectrum. The ordinates apply to the yellow data in the middle; those for orange and red have been raised 0.5 and 1.0 log units respectively, and those for green and blue have been lowered 0.5 and 1.0 log units respectively. Note the increasing size of the low intensity section with decreasing wavelength corresponding to the increasing sensibility of the rods in the short wave part of the spectrum. The curves through the data are all from the equation used for Fig 2.

The form which the data assume follows expectation. The measurements with red light show only a single continuous intensity discrimination function. The data with orange light, however, already show a slight break quite comparable to that shown by Koenig

and Brodhun's measurements, and the same is true of measurements with yellow light (*cf* Fig 7 in Hecht, 1935) The data as a whole show that the extent of the low intensity section steadily increases as the spectrum goes from red to blue, and this is in conformity with the Duplicity theory as implemented by the spectrum sensibility curves of rods and cones

## v

*Photochemical Theory*

In addition to their relation to the Duplicity theory, these measurements, in their quantitative implications, are consonant with the theory developed for intensity discrimination in vision and photo-reception (Hecht, 1935) Essentially, this theory supposes that in order to discriminate between an intensity  $I$ , and another just perceptibly brighter intensity,  $I + \Delta I$ , a constant increment in the photochemical decomposition must take place in a given time when the photosensory system in the receptor is exposed to the added intensity  $\Delta I$  Since the exposure to  $\Delta I$  is constant in these measurements, this is equivalent to saying that a constant initial photochemical change must be produced by  $\Delta I$ , in order that its addition to  $I$  will be just perceptibly recognized

Of the equations deduced in terms of such an interpretation, the one which fits the cones best by far is

$$\Delta I/I = c[1 + 1/(KI)^{1/2}]^2$$

and it is actually the curve for this equation which is drawn through all the cone data in Figs 2 and 3 This is the equation which originally (Hecht, 1935) was found to fit Blanchard's data, those of Steinhardt, of Koenig and Brodhun, and has since been found to describe the more recent data of Smith (1936) and of Graham and Kemp (1938) Its agreement with the present measurements is obvious

The same curve has been drawn through the measurements of the rod sections One cannot be too sure of the rod curves, because the data do not cover a large enough range Steinhardt's rod sections, as well as our own, are best fitted by this equation, however, this does not exclude a fair agreement with the other equations involving slightly different exponents

The double logarithmic grid on which the data are plotted is useful for comparison with theory, because on such a plot the form of the curve resulting from the equation is invariant. The constants  $c$  and  $K$  merely fix the position of the curve on the ordinates and abscissas respectively, and comparison between measurements and theory may be made by inspection without computing the numerical values of  $c$  and  $K$ . The constant  $c$  is obviously the asymptotic value of  $\Delta I/I$  at the highest intensities, and  $K$  is the reciprocal of the intensity at which  $\Delta I/I$  is four times as large as its minimal value.

It is to be noted that for the data with blue light, three points near the transition are distinctly off the theoretical curve. This phenomenon has already been noted with intermittent stimulation (Hecht and Schlaer, 1936). These aberrant points cannot be attributed to summation of the effects of rods and cones, since such a summation could just as well take place at the transition for white light, for green, and for yellow, but is not evident in any of these functions. There is some evidence that these points may represent the behavior of elements which have the spectrum visibility curve of rods but the threshold of cones, and are comparable to those described in the eye of a completely colorblind individual (Hecht, Schlaer, Smith, Haig, and Peskin, 1938).

#### SUMMARY

1 A new apparatus is described for measuring visual intensity discrimination over a large range of intensities, with white light and with selected portions of the spectrum. With it measurements were made of the intensity  $\Delta I$  which is just perceptible when it is added for a short time to a portion of a field of intensity  $I$  to which the eye has been adapted.

2 For white and for all colors the fraction  $\Delta I/I$  decreases as  $I$  increases and reaches an asymptotic minimum value at high values of  $I$ . In addition, with white light the relation between  $\Delta I/I$  and  $I$  shows two sections, one at low intensities and the other at high intensities, the two being separated by an abrupt transition. These findings are contrary to the generally accepted measurements of Koenig and Brodhun, however, they confirm the recent work of Steinhardt, as well as the older work of Blanchard and of Aubert. The abrupt transition is in keeping with the Duplicity theory which

attributes the two sections to the functions of the rods and cones respectively

3 Measurements with five parts of the spectrum amplify these relationships in terms of the different spectral sensibilities of the rods and cones With extreme red light the relation of  $\Delta I/I$  to  $I$  shows only a high intensity section corresponding to cone function, while with other colors the low intensity rod section appears and increases in extent as the light used moves toward the violet end of the spectrum

4 Like most of the previously published data from various sources, the present numerical data are all described with precision by the theory which supposes that intensity discrimination is determined by the initial photochemical and chemical events in the rods and cones

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# LIMITING FACTORS IN PHOTOSYNTHESIS LIGHT AND CARBON DIOXIDE

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## I

### INTRODUCTION

It was F. F. Blackman (1905) who first recognized that in photosynthesis where the "process is conditioned as to its rapidity by a number of separate factors, the rate of the process is limited by the pace of the 'slowest' factor"<sup>1</sup> In terms of this idea it was possible to identify two processes in photosynthesis, one, a photochemical reaction, and the other, a temperature sensitive (Blackman) reaction (Warburg, 1919, 1920, Emerson and Arnold, 1932), both involving chlorophyll in a cycle Using this cycle as a first approximation, kinetic descriptions have been developed for some of the properties of photosynthesis (e.g., Baly, 1935, Burk and Lineweaver, 1935, Smith, 1937) However, no complete description, either experimental or theoretical, has yet been given of the interrelationships of the different factors which may limit the photosynthesis rate

The present paper deals with light intensity and CO<sub>2</sub> concentration as limiting factors We intend first, to show that this relationship may be derived from the equations which we have used to describe other properties of photosynthesis, and second, to present a series of measurements which have been made to test the validity of these ideas

## II

### *Theoretical*

We have shown (Smith, 1937) that the measurements of photosynthesis rate ( $p$ ) as a function of light intensity ( $I$ ) or of CO<sub>2</sub> con-

<sup>1</sup> Full accounts of the controversy over Blackman's ideas are given by both Stiles (1925) and Spoehr (1926) in their monographs

centration at the stationary state can be described by the expression

$$p = k_1 I(a^2 - x^2)^{1/2} = k_2 [\text{CO}_2] a \quad (1)$$

where  $a$  may be regarded as representing the total concentration of chlorophyll, and  $x$  the amount of chlorophyll activated by light. The terms containing  $I$  and  $[\text{CO}_2]$ , describe the velocities of the light and dark processes. If  $x$  is eliminated and equation (1) is solved for  $p$  as a function of  $I$  at constant  $[\text{CO}_2]$ , or as a function of  $[\text{CO}_2]$  at constant  $I$ , we obtain equations which describe accurately the available data. In logarithmic form, these equations are

$$\log p = \log p_{ma} - 1/2 \log \left( 1 + \frac{1}{K_1^2 I^2} \right) \quad (2)$$

and

$$\log p = \log p_{mb} - 1/2 \log \left( 1 + \frac{1}{K_2^2 [\text{CO}_2]^2} \right) \quad (3)$$

where the maximum photosynthesis rates,  $p_{ma} = k_2 [\text{CO}_2] a$  and  $p_{mb} = k_1 I a$ ,  $K_1 = k_1/k_2 [\text{CO}_2]$  and  $K_2 = k_2/k_1 I$ . If  $\log p$  is plotted against  $\log I$  (or  $\log [\text{CO}_2]$ ), the shape of the curve obtained is independent of the constants  $K$  and  $p_m$ .

This curve is linear at low intensities, gradually curving to a maximum photosynthesis rate at high intensities. This maximum varies with the  $\text{CO}_2$  concentration. A precise way of determining the limiting conditions is to secure a family of curves relating photosynthesis and intensity at different  $\text{CO}_2$  concentrations and from them to find the relationship between the intensity and the  $\text{CO}_2$  concentration required to produce a definite photosynthesis rate. A family of  $\text{CO}_2$ -photosynthesis curves at different intensities can be treated similarly. If equation (1) has more than *ad hoc* value, it should be possible to predict from it the nature of the relationship to be expected.

Starting with equation (1),  $x$  may be eliminated by substituting  $p/k_2 [\text{CO}_2]$ . The expression is then solved for  $[\text{CO}_2]$  as a function of  $I$  when  $p$  is constant. This yields in logarithmic form

$$\log A_2 [\text{CO}_2] = -1/2 \log \left( 1 - \frac{1}{A_1^2 I^2} \right) \quad (4)$$

where  $A_1 = k_1 a/p$  and  $A_2 = I_0 a/p$ . Equation (4) may be plotted as  $\log [\text{CO}_2]$  against  $\log I$  giving a curve whose shape is independent of the constants  $A_1$  and  $A_2$  which define the asymptotes. Reversing the position of  $I$  and  $[\text{CO}_2]$  in the equation yields the same function, so that either variable can be considered as dependent or independent.

Equations similar to (4) but having somewhat different shapes may be obtained by changing the exponents in equation (1). Where the terms for the light and dark processes are those of a simple first order nature, as in

$$p = k_1 I(a - x) = k_2 [\text{CO}_2]x \quad (5)$$

solving at constant  $p$  yields

$$\log A_1 [\text{CO}_2] = -\log \left( 1 - \frac{1}{A_1 I} \right) \quad (6)$$

Where the exponents are second order, as in

$$p = k_1 I(a - x)^2 = k_2 [\text{CO}_2]x^2 \quad (7)$$

solving as before, gives

$$\log A_1 [\text{CO}_2] = -2 \log \left( 1 - \frac{1}{A_1^{1/2} I^{1/2}} \right) \quad (8)$$

The properties of equations (6) and (8) are similar to those of (4) and can be treated in the same way. The curves obtained from the three equations are drawn for comparison in Fig. 1. In addition to these three curves, many others of different curvature may be obtained by changing the exponents for the light and dark processes. Thus, all curves obtained on the basis of a two process cycle are in agreement with the idea of limiting factors, the form of the curve depends on the nature of the functions which describe the light and dark reactions.

The  $\log I$  and  $\log [\text{CO}_2]$  asymptotes represent the minima necessary to produce a definite photosynthesis value. When either of these two variables is greater than the necessary minimum, the magnitude of the other factor can be reduced accordingly, until finally its minimum is reached. Since the rate of curvature depends on the kinetic properties of the light and dark processes, information on this point can be obtained from the data. It should be emphasized that such data present information somewhat different from that given by an

investigation of the effect of a single variable For the effect of light intensity (or  $[\text{CO}_2]$ ) on photosynthesis, equations (1) and (4) give curves which have the same slope at low intensities The two equations differ only in their rate of curvature at high photosynthesis

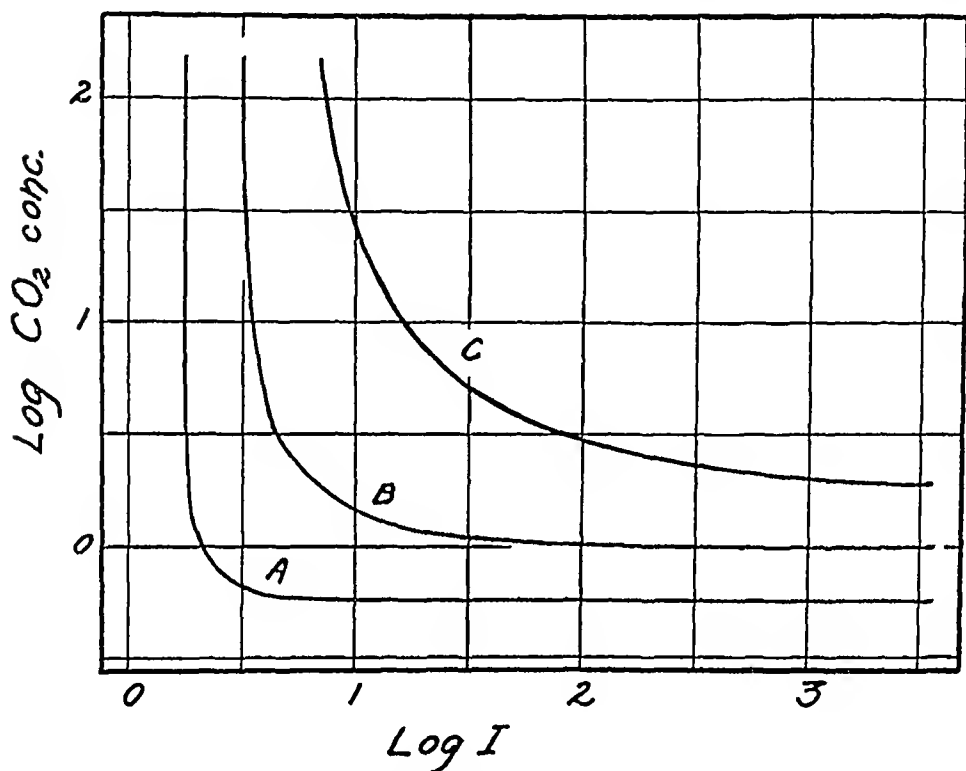


FIG 1 The relation between light intensity and  $\text{CO}_2$  concentration necessary for a constant amount of photosynthesis Curves A, B, and C represent equations (4), (6), and (8) The three curves are drawn to asymptotes 0.25 log units apart

values as they approach the maximum rate By using the data of  $\text{CO}_2$  concentration *versus* light intensity, the kinetics of the process can be independently evaluated at all measured values of the photosynthesis rate

### III

#### RESULTS

In order to test the theoretical curves developed in section II, it is necessary to have families of curves for photosynthesis at different  $\text{CO}_2$

concentrations and light intensities. The data for four different photosynthesis values taken from earlier measurements (Smith, 1936, 1937) are presented in Fig 2 and Table I. The curve for equation (4) has been drawn through the data.

Fig 2 shows good general agreement with the theoretical expectation at high photosynthesis values, but the range which these measurements cover yields insufficient information in the transitional region

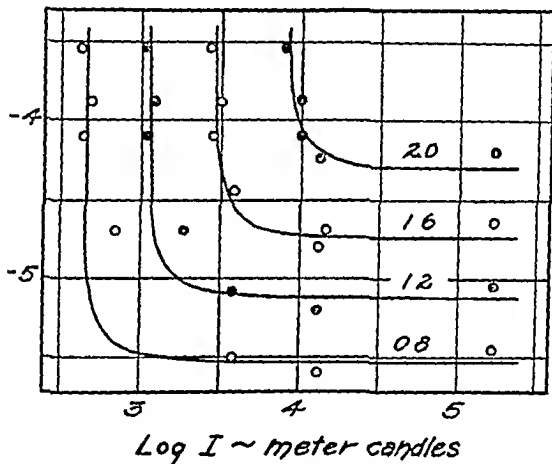


FIG 2 The intensity and  $\text{CO}_2$  concentration at four log photosynthesis values given by the numbers on the curves. The curve drawn is theoretical and is from equation (4). The data are taken from Smith (1937) and are in Table I.

between the asymptotes at low photosynthesis values, this is precisely where the least decisive evidence is given by the curves relating intensity (or  $[\text{CO}_2]$ ) and photosynthesis. In addition, these data are expressed in terms of wet weight of tissue, and there may be 20 to 30 per cent variation in photosynthesis rate, thus affecting the relative position of each curve on the ordinate.

A new series of measurements to eliminate these two objections was

therefore undertaken. The photosynthesis of the fresh water plant *Cabomba caroliniana* was studied as in the previous investigation, using the Warburg apparatus with the same methods for the control of light intensity and  $\text{CO}_2$  concentration. All of the measurements were made at  $25^\circ\text{C}$ .

In order to eliminate the variation caused by the use of different fronds, a measurement of the photosynthetic activity of each frond was made under standard conditions  $[\text{CO}_2] = 2.90 \times 10^{-4}$  moles per liter (Warburg buffer No. 11),  $I = 123,000$  meter candles. All of the

TABLE I

*Intensity and  $\text{CO}_2$  Concentration for Constant Photosynthesis*

These data drawn in Fig. 2 represent interpolated values from the measurements given in Tables III and IV of an earlier publication (Smith, 1937). The intensities in Table IV of that paper have been corrected for the absorption of the red filter (Corning No. 246) as determined by measuring photosynthesis-intensity curves on the same plant with white and with red light. The effective absorption of the filter as determined twice was 0.22 log units. Bold-face values are for the factor that was constant in the measurements.

$\log p = 0.8$		$\log p = 1.2$		$\log p = 1.6$		$\log p = 2.0$	
$\log [\text{CO}]$	$\log I$	$\log [\text{CO}]$	$\log I$	$\log [\text{CO}]$	$\log I$	$\log [\text{CO}_2]$	$\log I$
-5.50	3.58	-5.08	3.58	-4.45	3.58	—	—
-5.59	4.12	-5.20	4.12	-4.80	4.12	-4.24	4.12
-5.45	5.23	-5.05	5.23	-4.64	5.23	-4.20	5.23
-4.69	2.84	-4.69	3.27	-4.69	4.17	—	—
-4.10	2.63	-4.10	3.03	-4.10	3.45	-4.10	4.00
-3.88	2.68	-3.88	3.08	-3.88	3.49	-3.88	4.00
-3.54	2.62	-3.54	3.02	-3.54	3.43	-3.54	3.90

data were then corrected in terms of an assigned arbitrary photosynthesis value of 200 c. mm. of oxygen produced per hour per 100 mg. wet weight of tissue for the standard determination. This value is within 5 per cent of the average actually found.

To cover a sufficient range, measurements were made at five light intensities and six  $\text{CO}_2$  concentrations. Within a single experiment, the photosynthesis of a frond was investigated as a function of light intensity at a constant  $\text{CO}_2$  concentration, and then repeated for one or two additional  $\text{CO}_2$  concentrations. Three runs were made at

each  $\text{CO}_2$  concentration, a total of eighteen for the series, and the data averaged. While the data were all obtained as photosynthesis at different intensities, they may also be used to obtain the  $\text{CO}_2$  curves at constant intensity. Two complete series of such measurements were made, they are presented in Table II.

To find the light intensity necessary to attain a definite amount of photosynthesis at a constant  $\text{CO}_2$  concentration, or the converse, it is

TABLE II

*Photosynthesis at Different Intensities and  $\text{CO}_2$  Concentrations*

Data of Figs 3 and 4. Photosynthesis in cubic millimeters of oxygen evolved per hour per 100 mg wet weight of material corrected for respiration. Temperature =  $25.3^\circ\text{C}$ .  $\text{CO}_2$  concentrations  $\times 10^6$  in moles per liter. All of the data are in terms of a standard value of 200 when the  $[\text{CO}_2] = 290 \times 10^{-6}$  and  $I = 123,000$  meter candles. Each set of data represents the averages of three similar experiments.

Series	Intensity  meter candles	Photosynthesis rate					
		$[\text{CO}_2] = 4.48$ Buffer No 5	$[\text{CO}_2] = 8.67$ Buffer No 6	$[\text{CO}_2] = 20.5$ Buffer No 7	$[\text{CO}_2] = 37.5$ Buffer No 8	$[\text{CO}_2] = 78.7$ Buffer No 9	$[\text{CO}_2] = 290$ Buffer No 11
I	407	2.89	3.03	3.03	3.36	4.08	3.66
	1,740	7.59	9.62	13.1	16.5	17.0	18.7
	6,310	9.75	19.2	33.7	45.9	62.1	62.5
	21,900	9.91	21.0	43.8	66.4	119	150
	123,000	10.4	21.1	46.5	71.5	145	200
II	407	2.06	3.21	3.40	3.74	3.67	4.49
	1,740	7.16	11.2	14.2	19.5	17.4	20.5
	6,310	10.8	20.4	35.9	54.7	55.2	68.4
	21,900	11.9	23.2	48.3	79.1	119	159
	123,000	11.6	23.5	48.4	84.9	146	200

necessary to interpolate between the measured values. To do this, there was drawn through all of the data, the smooth curve of equation (2). That this curve gives a satisfactory description of these data is shown in Figs 3 and 4. In Fig 3 are presented the data of series I for photosynthesis as a function of intensity. All of the data have the curve of equation (2) drawn through them.

The mass plots of Fig 4 contain all of the data in Table II. The



curve of equation (2) was drawn through all of the log photosynthesis *versus* log  $I$  data at the different  $\text{CO}_2$  concentrations. These curves

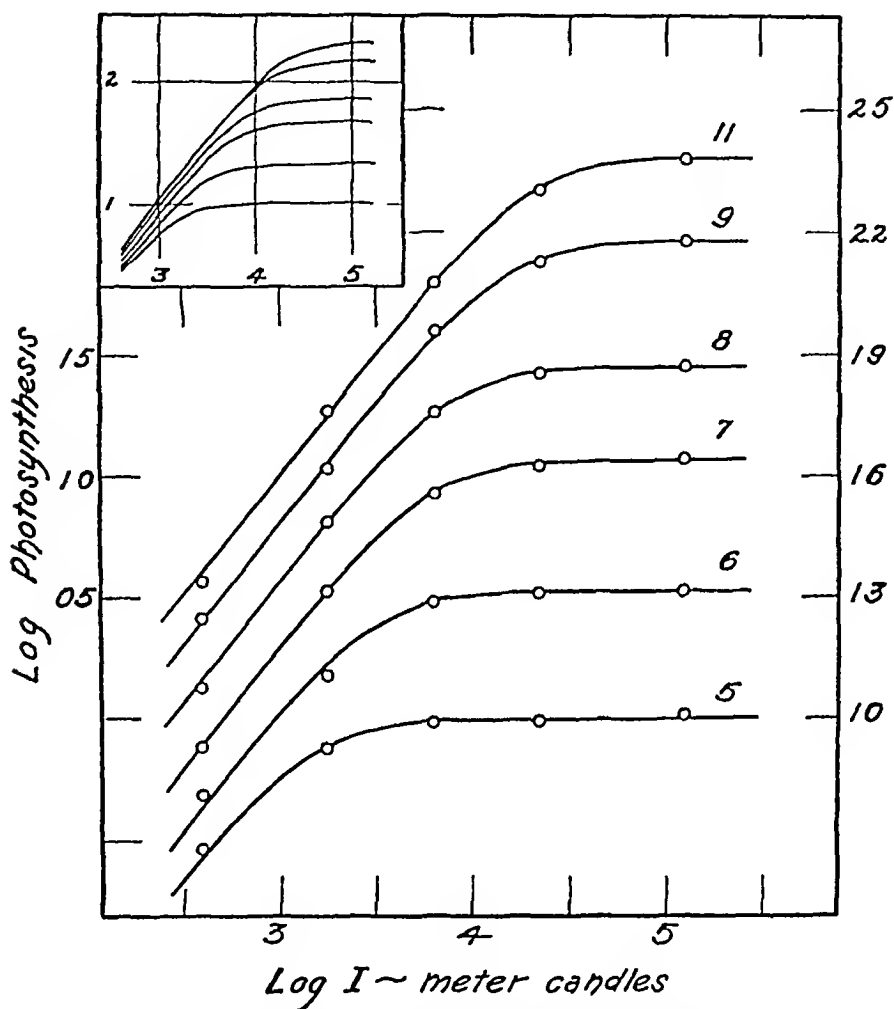


FIG 3 Photosynthesis as a function of light intensity for different  $\text{CO}_2$  concentrations indicated on each curve by the Warburg buffer number. The photosynthesis ordinates are correct only for the uppermost curve, the others have been displaced downwards in steps of 0.2 of a log unit, with their correct positions given on the right side of the figure. The insert shows the absolute positions of the six curves drawn to exactly half the ordinates. All of the curves are drawn from equation (2). These are from the data of series I given in Table II.

were then superimposed and the points traced on a single graph. This is possible because the shape of the curve is invariant in form.

The same procedure was also used for the log photosynthesis—log  $\text{CO}_2$  data, except for the measurements at the lowest light intensity (407 meter candles) which are omitted because they were not sufficiently precise to determine the position of the curve. The excellent fit of these new data incidentally confirms and strengthens the validity of equations (2) and (3) as quantitative descriptions of the effect of these two variables on photosynthesis.

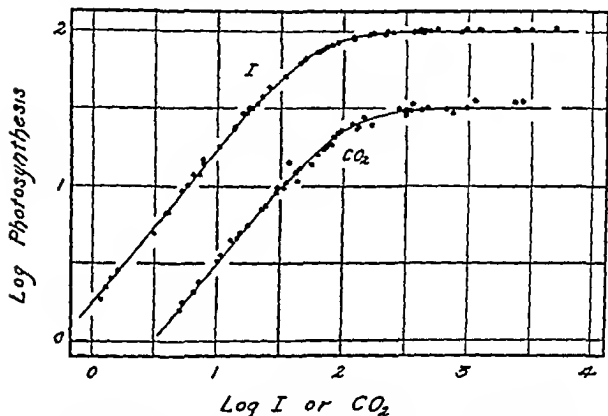


FIG. 4. Photosynthesis as a function of intensity (upper curve) and of  $\text{CO}_2$  concentration (lower curve). The points represent all of the data in Table II. The curve from equation (2) was drawn through all of the different sets of data. These were then superimposed and traced on a single graph.

In Fig. 5 and Table III are presented the data obtained for the log  $\text{CO}_2$  concentration—log intensity relationship at four log photosynthesis values. These four values were chosen to express best the actual data obtained. The lowest photosynthesis value is near the lower limit of the actual measurements, and in a few cases represents a small extrapolation. The higher values of the log photosynthesis rate were selected to eliminate successively one or more curves from consideration, thus giving whatever real changes in form occur in low as compared with higher photosynthesis rates.

Equation (4) has been drawn through the data in Fig 5. A good description of the data is obtained at the three higher photosynthesis rates, as is also the case for the older data given in Fig 2. However, at the lowest photosynthesis value, a much better description is given by equation (6), which is drawn in broken lines.

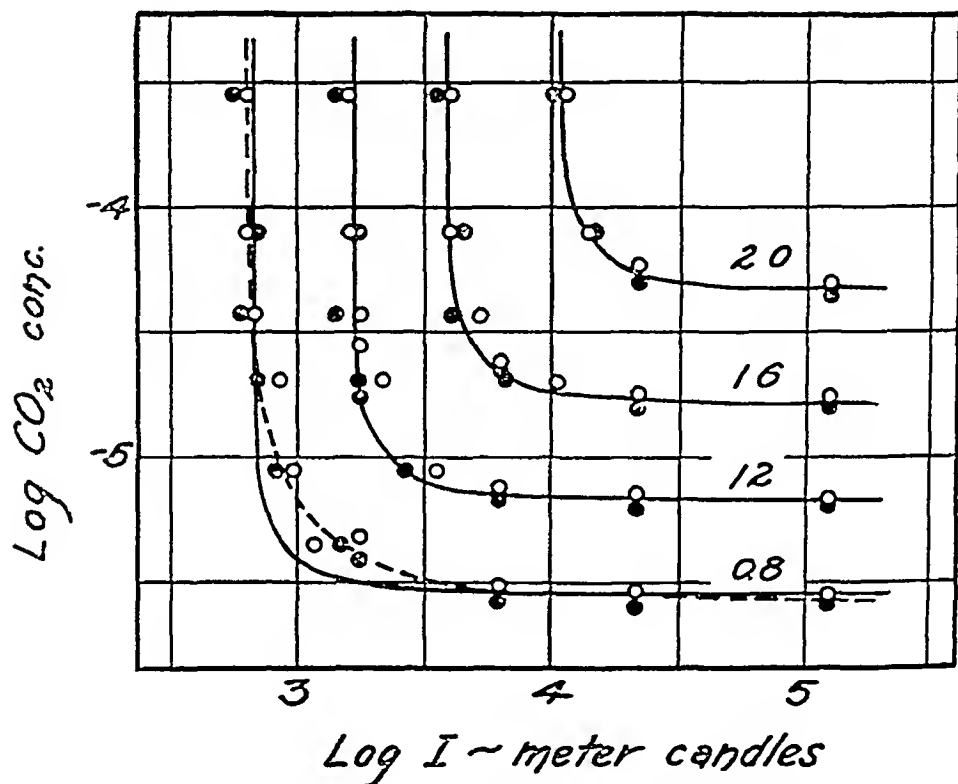


FIG 5 Intensity and  $\text{CO}_2$  concentration at four log photosynthesis values indicated on each curve. The curves are theoretical and represent equation (4). At the lowest photosynthesis value, a better fit of the data is given by equation (6) indicated by dashed lines. The open circles are from series I, and the solid circles are from series II. The numerical values are given in Table III.

After the first series of measurements had been completed, it was thought that the difference in curve form might be due to large errors or variation at low photosynthesis rates, and it was for this reason that a second series of measurements was undertaken. The data show no significant difference between the two series. The average difference in the determination of  $\log I$  or of  $\log [\text{CO}_2]$  between the two

series is about 0.07 log units. This value is actually an exaggeration of the uncertainty in placing the curve, because there is a distinct shift in the best position of the curves independently drawn through the two series. This shift is about 0.05 log units with respect to both

TABLE III

*Intensity and CO<sub>2</sub> Concentration for Constant Photosynthesis*

Data of Fig. 5. These are graphically interpolated values from the measurements in Table II. The numbers in bold face type represent the constant factor

Series	log $p = 0.30$		log $p = 1.20$		log $p = 1.60$		log $p = 2.00$	
	log [CO <sub>2</sub> ]	log $I$	log [CO <sub>2</sub> ]	log $I$	log [CO <sub>2</sub> ]	log $I$	log [CO <sub>2</sub> ]	log $I$
I	-5.32	<b>3.24</b>	-4.55	<b>3.24</b>	—	—	—	—
	-5.52	<b>3.80</b>	-5.12	<b>3.80</b>	-4.62	<b>3.80</b>	—	—
	-5.54	<b>4.34</b>	-5.14	<b>4.34</b>	-4.74	<b>4.34</b>	-4.23	<b>4.34</b>
	-5.56	<b>5.09</b>	-5.17	<b>5.09</b>	-4.76	<b>5.09</b>	-4.31	<b>5.09</b>
	-5.35	<b>3.07</b>	—	—	—	—	—	—
	-5.06	<b>2.94</b>	-5.06	<b>3.55</b>	—	—	—	—
	-4.69	<b>2.92</b>	-4.69	<b>3.31</b>	-4.69	<b>4.02</b>	—	—
	-4.43	<b>2.83</b>	-4.43	<b>3.24</b>	-4.43	<b>3.72</b>	—	—
	-4.10	<b>2.80</b>	-4.10	<b>3.20</b>	-4.10	<b>3.61</b>	-4.10	<b>4.14</b>
	-3.54	<b>2.80</b>	-3.54	<b>3.20</b>	-3.54	<b>3.60</b>	-3.54	<b>4.06</b>
II	-5.41	<b>3.24</b>	-4.75	<b>3.24</b>	—	—	—	—
	-5.58	<b>3.80</b>	-5.17	<b>3.80</b>	-4.66	<b>3.80</b>	—	—
	-5.61	<b>4.34</b>	-5.21	<b>4.34</b>	-4.81	<b>4.34</b>	-4.30	<b>4.34</b>
	-5.59	<b>5.09</b>	-5.20	<b>5.09</b>	-4.80	<b>5.09</b>	-4.35	<b>5.09</b>
	-5.35	<b>3.17</b>	—	—	—	—	—	—
	-5.06	<b>2.92</b>	-5.06	<b>3.43</b>	—	—	—	—
	-4.69	<b>2.84</b>	-4.69	<b>3.24</b>	-4.69	<b>3.82</b>	—	—
	-4.43	<b>2.77</b>	-4.43	<b>3.16</b>	-4.43	<b>3.61</b>	—	—
	-4.10	<b>2.84</b>	-4.10	<b>3.24</b>	-4.10	<b>3.65</b>	-4.10	<b>4.17</b>
	-3.54	<b>2.75</b>	-3.54	<b>3.15</b>	-3.54	<b>3.55</b>	-3.54	<b>4.01</b>

ordinates and may be due to some specific difference in the plants used, since the two series were run some 6 months apart.

It is possible that the curve obtained at low photosynthesis represents some specific kinetic difference as compared with those at high photosynthesis values. If this is so, then the curves for photosynthesis as a function of intensity should have different shapes at low and at high CO<sub>2</sub> concentrations, or the photosynthesis-CO<sub>2</sub> curves should

vary with light intensity. The published data on these relationships (Smith, 1936, 1937) as well as those given in this paper do not show such variation except where a  $\text{CO}_2$  diffusion factor is involved. Such measurements, however, have been usually made with the constant factor at moderate or high values. In order to test this possibility, new measurements were undertaken.

For the photosynthesis-intensity measurements, a buffer (No. 2) of low  $\text{CO}_2$  concentration was selected which gave measurements of photosynthesis below the compensation point even at high intensities.

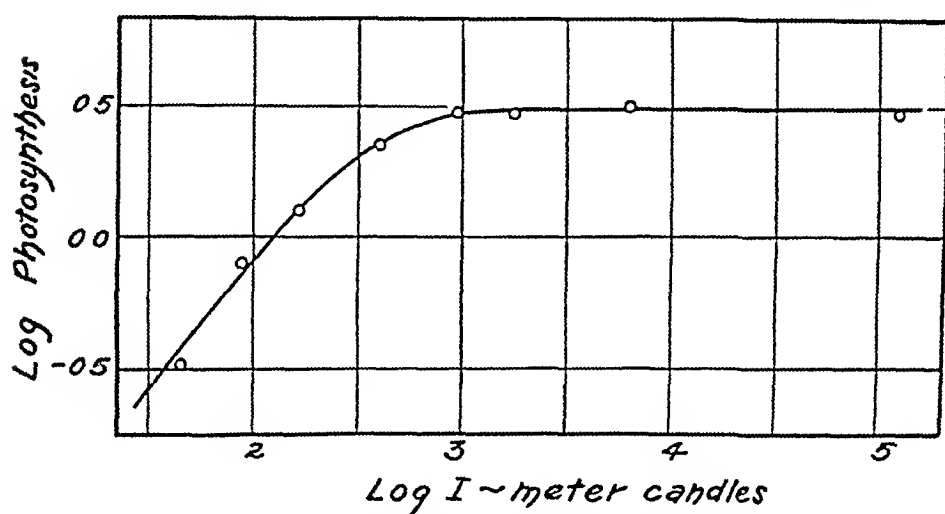


FIG. 6. Photosynthesis as a function of intensity at a low constant  $\text{CO}_2$  concentration ( $0.902 \times 10^{-6}$  moles per liter). The curve is from equation (2) and is of the same form as those obtained at higher  $\text{CO}_2$  concentrations. The data are given in Table IV.

For such low photosynthesis rates, the readings had to be of long duration, respiration was measured for at least 1 hour, and the determinations at each light intensity for 30 minutes. Three fronds were used in each run to increase the accuracy of the measurements.

The data which are given in Fig. 6 and Table IV represent the averages of three similar runs. These measurements are adequately described by equation (2) and therefore do not differ from the results obtained when higher  $\text{CO}_2$  concentrations are used.

It is more difficult to determine the curve for photosynthesis as a function of  $\text{CO}_2$  concentration at low intensity. The tissue has to be

removed from the manometer vessel after each determination and replaced in a buffer mixture of a different  $\text{CO}_2$  concentration. Such handling has little effect on measurements made at high intensities, but at low intensities it is sufficient to invalidate the measurements obtained. Moreover, one cannot use several fronds because they cannot be replaced in the vessel without altering the overlapping and partial shading. The form of the photosynthesis- $\text{CO}_2$  curve at low intensities can be determined only with the use of more suitable material.

TABLE IV

*Photosynthesis and Intensity at Low  $\text{CO}_2$  Concentration*

Data of Fig. 6. Measurements at constant  $\text{CO}_2$  concentration =  $0.902 \times 10^{-6}$ , Buffer No. 2. Averages of three similar runs using three large fronds in each run. Temperature =  $25.3^\circ\text{C}$ . Respiration determined for at least 60 minutes, other measurements for 30 minutes each. Photosynthesis in cubic millimeters of oxygen per hour per 100 mg. wet weight of tissue.

Intensity	Photosynthesis
<i>meter candles</i>	
45.7	0.32
87.1	0.79
166	1.26
407	2.27
933	2.94
1,740	2.93
6,310	3.13
123,000	2.96

## IV

## DISCUSSION

In addition to the data presented in this paper, there have been only two attempts to obtain a comprehensive picture of the mutual effects of intensity and  $\text{CO}_2$  concentration on photosynthesis, those of Harder (1921) on *Fontinalis*, and those of Hoover, Johnston, and Brackett (1933) on wheat. We have studied their results in the same way that we have done for *Cabomba*.

For the higher photosynthesis values of Harder's measurements, the uncertainty in drawing the individual curves makes difficult a

choice between equations (4) and (6), but at the low values, equation (6) definitely gives a better fit than (4). This is entirely consistent with the results on *Cabomba*.

The two experiments of Hoover, Johnston, and Brackett present a somewhat different picture. The data for their first experiment give curves which change their shape at low and high photosynthesis values in much the same way as those for *Cabomba* and *Fontinalis*. However, for reasons which are at present obscure, the measurements for their second experiment differ considerably from all of the other data. Here the curvature in the  $\log \text{CO}_2$  versus  $\log$  intensity graphs becomes very gradual and fits equation (8) best.

The explanation for the difference in the limiting factor equations at low and high photosynthesis rates depends on the interpretation of the exponents in these equations. Franck and Herzfeld (1937), after assuming the existence of back-reactions in photosynthesis, arrived at an equation similar to equation (2). While equation (2) gives a slightly more precise description of the data than does their equation, the latter does provide a possible explanation of the change in curve form at high photosynthesis rates in terms of an appreciable back-reaction at high intensities and a negligible back-reaction at low intensities. In the latter situation, the light-limiting reaction (assuming that there must be four) would be first order, and a scheme such as given by equation (5) would hold. With appreciable amounts of energy loss caused by back-reaction, the data would fit the descriptions given by (1).

Whatever may be the eventual explanation for this change in kinetic properties at low and high photosynthesis rates, the general form of the limiting factor relationship seems clear. This relationship follows from the relative effects of the light and dark processes in the photosynthetic cycle, in fact, such a relationship must obtain whenever such a cycle occurs.

#### SUMMARY

1 Extensive measurements have been obtained (a) relating photosynthesis and light intensity for a large range of  $\text{CO}_2$  concentrations and (b) relating photosynthesis and  $\text{CO}_2$  at different light intensities. From these families of curves, the limiting factor relationship can be secured for any value of the photosynthesis rate.

2 In terms of previous work an equation has been derived for describing these relations between the intensity and  $\text{CO}_2$  concentration necessary to produce a definite amount of photosynthesis. This equation furnishes an exact description for all the data, except those for low rates of photosynthesis where a slightly different equation is required. The nature of the two equations suggests that a simple first order reaction determines the velocity of the light process at low photosynthesis rates, but that at high rates the mechanism is complicated by another factor.

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# ELECTRIC IMPEDANCE OF NITELLA DURING ACTIVITY\*

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## INTRODUCTION

There are many theories of the nature and mechanism of the processes of excitation, conduction, and recovery in irritable cells, but those which emphasize the electrical aspects of these phenomena are the most attractive and probably the most important because these effects can be measured with a high degree of precision and speed.

It is rather commonly accepted that the process of excitation of irritable cells starts as a more or less localized depolarization of the cell membrane. When the adjacent resting regions are then depolarized by the resultant current flow, the process continues as a propagated wave.

It is not surprising that both the cause and the course of the depolarization have remained vague and uncertain when most of the information has come from electrical excitation and action potential studies while the properties of the resting membrane have not been well understood. Consequently the attempts to express this picture in more quantitative form have usually encountered so many unknown factors that it has been necessary to use either poorly defined concepts or a considerable number of assumptions.

Two outstanding unknowns have been the electrical resistance and capacity of the membrane and the resistance of the cell interior. From longitudinal direct current resistance measurements on resting *Nitella*, Blinks (1930) obtained values of  $10^5 \Omega \text{ cm}$  or more which we may interpret as an ohmic membrane resistance. Since similar data by Rosenberg and Schnauder (1923) on the frog sciatic give  $4 \cdot 10^4 \Omega \text{ cm}^2$ , we may expect that such a resistance is not peculiar to *Nitella*.

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In common with a number of other types of cells, resting membrane capacities of approximately  $1\mu\text{f}/\text{cm}^2$  have been found for muscle and nerve (Cole and Curtis, 1936), *Nitella* (Curtis and Cole, 1937), and the squid giant axon (Curtis and Cole, 1938). These membrane capacities have associated with them a loss, similar to that found in common dielectrics, giving them a phase angle of less than  $90^\circ$ , which is independent of frequency.

There is less information as to the effect of excitation on these quantities. Lullies (1930) reported a decrease in the longitudinal low frequency impedance of nerve, and Blinks (1936) showed a loss of polarizability (the change of potential difference across the membrane due to current flow from an external source) in *Nitella*. In these experiments, it was not possible to separate the membrane resistance and capacity and follow the time course of each during excitation, so that a different method of approach is necessary.

In the present work, the impedance properties of *Nitella* have been measured, transverse to the cell axis, and over a wide frequency range, during the passage of an impulse, with simultaneous records of the cell action potential under the impedance electrodes. These data give the time course of the resistance, capacity, and potential changes of the membrane during activity and so provide a more complete and quantitative description of the impulse than has been possible before.

*Nitella* was chosen because it has large single cells, lives in fresh water, and is quite slow in its responses. The behavior of this plant cell is analogous to that of nerve in so many respects that one might also expect that the processes of activity would be found to be similar. The structure of *Nitella* is apparently more complicated than that of nerve and has been the cause of some difficulty in the interpretation of the impedance properties of the cell at rest (Curtis and Cole, 1937) and in activity. As has been pointed out, there are two protoplasmic interfaces in *Nitella*, but since our measurements have not given any evidence which provides a means for separating the properties of the two surfaces, we shall consider the observed characteristics to be due to a single membrane.

The alternating current transverse impedance technique which has been used for the resting *Nitella* and other cells is a relatively simple and straightforward method for determining the electrical

properties of the membrane and cell interior. In order to use it for the present problem, the methods of measurement and interpretation have had to be extended considerably.

### Material and Measuring Cell

*Nitella flexilis*, Ag, was grown in an aquarium which was balanced with guppies and radiated periodically with a neon lamp.

To obtain satisfactory action potentials,  $M/1000$   $KNO_3$ ,  $M/10,000$   $KH_2PO_4$ , and  $M/10,000$   $MgSO_4$  were added to the tap water in the aquarium. The *Nitella* grew quite readily under these conditions, and only healthy, growing cells were used for the experiments.

The cell chosen was cut away from its neighbors and immediately placed in the measuring apparatus. About half an hour was allowed for equilibration before the experiment was started. The measuring cell is essentially the same as that previously used, and is shown in Fig. 1. It is made of glass, de Khotinsky

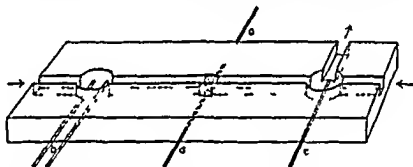


Fig. 1. Measuring cell for *Nitella*, with impedance electrodes, *aa*, stimulating electrodes, *b*, and action potential electrode, *c*. Arrows indicate the directions of flow of electrolytes.

cement, and various platinized platinum electrodes. The groove in which the *Nitella* cell was placed is 0.53 mm by 0.55 mm and 2 cm long, with a depression at each end to accommodate the cut ends of neighboring cells. The impedance electrodes, *aa*, were 0.5 mm long and were inlaid in opposite faces of the groove. This electrode length represents a compromise between the needs for measurements over as short a length of cell as possible, and a large electrode area to minimize electrode polarization corrections. The stimulating electrodes *b*, were placed at one end and an action potential recording electrode, *c*, at the other. Capillary siphons were placed at the ends of the grooves as indicated in Fig. 1. Aquarium water or  $M/1000$   $KCl$  was siphoned in at the stimulating electrode end, past the stimulating and impedance electrodes and out through the side tube. To obtain monophasic action potentials,  $M/10$   $KCl$  was siphoned in at the other end and out through the side tube, so that it bathed only the end of the *Nitella* under the recording electrode. This concentration of  $KCl$  was used to abolish the membrane activity because the cells seemed to last longer with  $KCl$  than with

chloroform In this way, good monophasic action potentials were recorded, and most cells would give reproducible results for many hours and some even for days The relatively refractory period for *Nitella* lasts from 3 to 5 minutes under the most favorable conditions, and it was necessary to allow this interval between stimuli, otherwise both the "resting" impedance and impedance change become progressively smaller

### *Electrical Equipment*

The impedance electrodes of the measuring cell were connected to the alternating current Wheatstone bridge (Cole and Curtis, 1937) and measurements of the parallel resistance and capacity (Cole, 1933, Cole and Curtis, 1936) were made from 0.05 to 100 kc (kilocycles per second) The amplified output of the bridge was applied to the vertical deflection plates of a cathode ray oscillograph A portion of the input oscillator voltage was applied to the horizontal deflection plates in such phase relation to the bridge output that a parallel resistance unbalance of the bridge caused the horizontal balance line to tip, while a parallel capacity unbalance alone gave an ellipse with a horizontal axis

The action potentials were led off between either the midpoint of a high resistance shunting the impedance electrodes, or the grounded impedance electrode, and the recording electrode at the inactive end of the cell These potentials were applied to the vertical deflection plates of a second cathode ray oscillograph through a differential direct current amplifier with degeneration in the common mode Both oscillographs are photographed simultaneously with a motion picture camera at from 17 to 64 frames per second Since motion pictures could be taken, a sweep circuit was unnecessary, and the action potential was recorded as a deflection from the base line on each picture

The stimulating current was obtained from a condenser discharge which had a time constant of about 1 millisecond Under these conditions it usually took about 30 volts to stimulate The stimulating circuit was electrically isolated from the rest of the circuits to reduce the stimulus artifact and considerable care had to be taken to avoid stimulation or injury by static charges

### *Procedure*

The single *Nitella* cell is placed in the trough of the measuring cell, covered, and the circulation of both aquarium water and KCl started When the impedance and resting potential have reached steady values, the bridge is balanced at one frequency and the amplifiers adjusted so that the maximum changes after stimulation remain on scale The camera is started as the cell is stimulated, and the entire action, lasting several seconds recorded Calibration figures are then recorded for known unbalances of both resistance and capacity This procedure is followed at each of the nine frequencies from 0.05 to 20 kc The cell is removed and a frequency run taken on aquarium water alone to obtain data for the capacity zero and electrode polarization (Cole and Curtis, 1937) In this way, a complete frequency run could be taken in 40 minutes

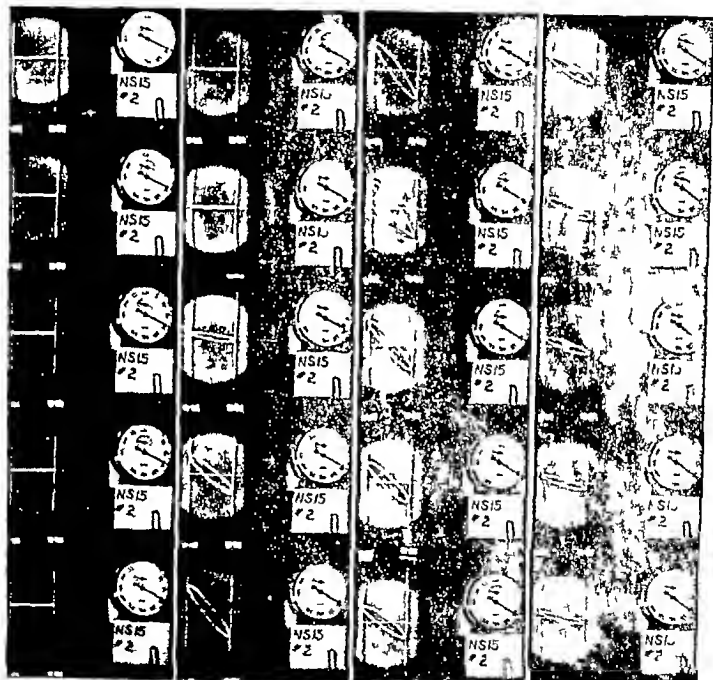


FIG 2 Section of a motion picture record of the bridge unbalance ellipses (left) action potential spot (center) and stop watch (right) following stimulation

A short section of a film taken at 0.2 kc and 17 frames per second is reproduced in Fig 2. The stimulus artifact can be seen in the first picture, and the action potential starts to rise immediately afterwards. When the action potential has nearly reached its maximum the bridge goes off balance rather suddenly and then both subside quite gradually.

The developed film is projected and the impedance ellipses and the action potentials are measured on the screen. From the dimensions of the experimental and calibration ellipses the changes in the parallel resistance and capacity are calculated by equations (3). The impedance at rest and at the time of each picture after stimulation

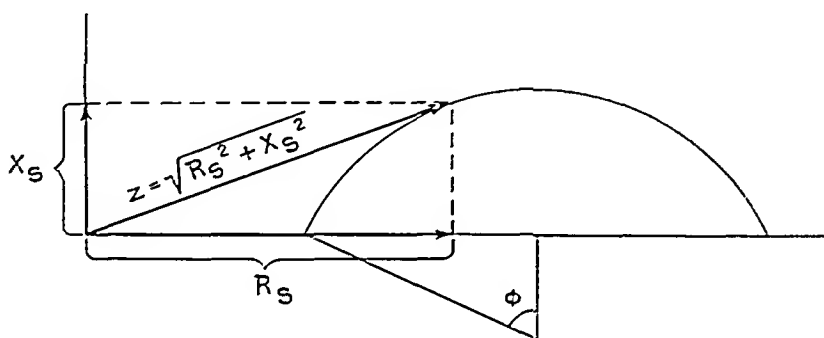


FIG 3 Schematic impedance locus, showing an impedance vector,  $z$ , with its two components, the series resistance,  $R_s$ , and series reactance,  $X_s$ .  $\phi$  is the membrane phase angle.

is computed as though the measurements had been made with the resistance,  $R_s$ , and the reactance,  $X_s$ , in series, by the equations

$$R_s = R_p / (1 + R_p^2 C_p^2 \omega^2) \quad \text{and} \quad X_s = R_p^2 C_p \omega / (1 + R_p^2 C_p^2 \omega^2)$$

$R_s$  and  $X_s$  are plotted to give the impedance locus as shown in Fig 3. With the cell at rest the end of the impedance vector follows a circular arc as the frequency is changed and this arc is called the frequency impedance locus. During the process of excitation the impedance at each frequency changes so that the tip of the impedance vector follows a path, such as  $D$  in Fig 4, which is called the time impedance locus.

## RESULTS

*Impedance*—It was found that there was some uncertainty as to the form of the frequency impedance locus at the time of maximum

change. This was probably due to an uncontrolled variation in the cell since the maximum change at any one frequency varied somewhat during an experiment. Since it was not possible to measure all frequencies during a single excitation, three excitations at each frequency were recorded in random order and the minimum impedances plotted in Fig. 4. It is quite apparent, in the first place, that there is no change in the extrapolated high frequency resistance. This means that the resistance of the interior of the cell, the resistance of the external fluid, and the volume concentration remain unchanged, or

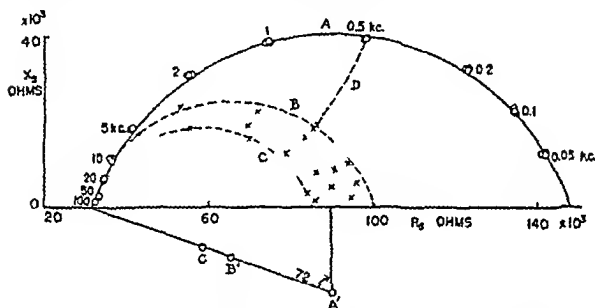


FIG. 4. Frequency impedance loci,  $\lambda$  series resistance,  $R_s$  series reactance,  $X_s$ . A, for *Nitella* at rest (O) and B and C for the extreme values of the maximum impedance changes during activity (X), with centers at A', B', and C'. Frequencies are given in kilocycles (kc.), and each frequency was repeated three times in random order.

else that any two, or all three, change in such a way that the infinite frequency resistance is unchanged. It is more reasonable to assume that there is no change in any of these quantities. In the second place, since the minimal excitation impedance points (X) are distributed rather well between two circular arcs representing the same phase angle as the resting cell, there is no indication of a change in the phase angle of the membrane at the height of the excitation and it will be assumed as a first approximation that there is no change of this membrane parameter during the course of the action. It will be shown that the maximum variability of the impedance changes shown in Fig. 4 rep



resents a  $\pm 10$  per cent maximum deviation of the membrane impedance from its mean value

During single excitations, the time course on the impedance locus has been plotted for three frequencies in Fig 5. Equation (4) shows that if the membrane capacity alone were to change during excitation, the zero frequency extrapolation would remain unchanged and the impedance at each frequency would merely move along the resting circle. Since this is not the case, and since the membrane phase angle, the internal and external resistances, and the volume of the cell (see

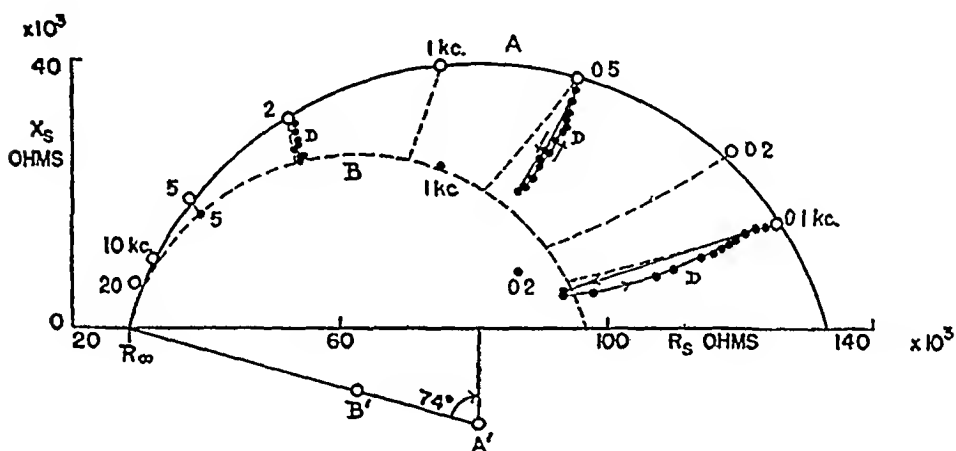


FIG 5 Impedance loci, *i.e.* series resistance,  $R_s$ , vs series reactance,  $X_s$ . The frequency loci are A, for *Nitella* at rest (○), and B, at height of activity with centers at A' and B'. The time loci, D, show how the impedance changes at 0.1, 0.5, and 2.0 kilocycles when the cell is stimulated (●). As indicated by the arrows, the time impedance locus during recovery does not exactly retrace the path of the initial change.

above) change little if at all, we must now consider the effect of a change in the membrane conductance or leakage.

The membrane conductance is thought of as due to a resistance in parallel with the membrane capacity. This conductance, which may be the representation of the ionic membrane permeability, is considered to be independent of frequency. On this assumption, it will be shown in equation (8) that if the membrane conductance alone were to change during the action, the time impedance locus would be a circular arc tangent to the resistance axis at the infinite frequency.

point ( $R_{\infty}$ ), as indicated by the dotted lines in Fig 5 and the arc C in Fig 11. This assumption gives an approximate explanation of the observations but it is found that the experimental points quite consistently fall to one side of the arc. The deviation may be interpreted as an average maximum decrease in the membrane capacity of 15 per cent from the resting value of  $0.9 \mu\text{f}/\text{cm}^2$ . This capacity change follows a time course similar to that of the membrane conductance. The two are slightly different, however, because it can be seen on the time impedance locus that, during recovery, the impedance does not exactly retrace the path of the initial change.

In the previous *Nitella* paper (Curtis and Cole, 1937) it was shown that, within experimental error, the conductance of the resting cell membrane was so small as to be negligible in transverse impedance measurements. The membrane conductance during excitation, calculated from data at 0.05 kc by equation (6), has been plotted in Fig 6 as a function of time. The maximum conductance in this case corresponds to a membrane resistance of  $400 \text{ ohm cm}^2$  with an average value for all experiments of  $500 \text{ ohm cm}^2$ . In the data of Fig 4, the maximum (B) and minimum (C) circles correspond to membrane resistances of 350 and  $287 \text{ ohm cm}^2$  or a  $\pm 10$  per cent fluctuation from the mean value for this typical *Nitella*. Furthermore, it is found that the time courses of the membrane conductances calculated from the data at frequencies shown in Fig 5 agree closely with each other, thus supporting the assumption that the membrane resistance is independent of frequency.

The observed rise of conductance is considerably faster than that of the action potential. However, if a sharp, or discontinuous change of conductance were to move past the 0.5 mm long impedance electrodes at a velocity of  $1.0 \text{ cm/sec}$  the observed time of rise of conductance should be at least 0.05 second. Actually, this time would be still longer because of the "fringing" of current at each end of the electrodes which increases their apparent length. It is simpler to obtain the apparent length from a model experiment than by calculation. A round glass rod was placed in the measuring cell instead of the *Nitella* and resistance measurements were made for a number of positions as the rod was moved to the right and its left hand end passed between the impedance electrodes. The end then corre

sponded to an abrupt transition from a non-conducting to a perfectly conducting membrane, although the difference between the measured resistances with and without the rod may not be large, as is seen from equation (4) The only data taken before this glass impedance cell was broken are those of Fig 7, but they show quite clearly that

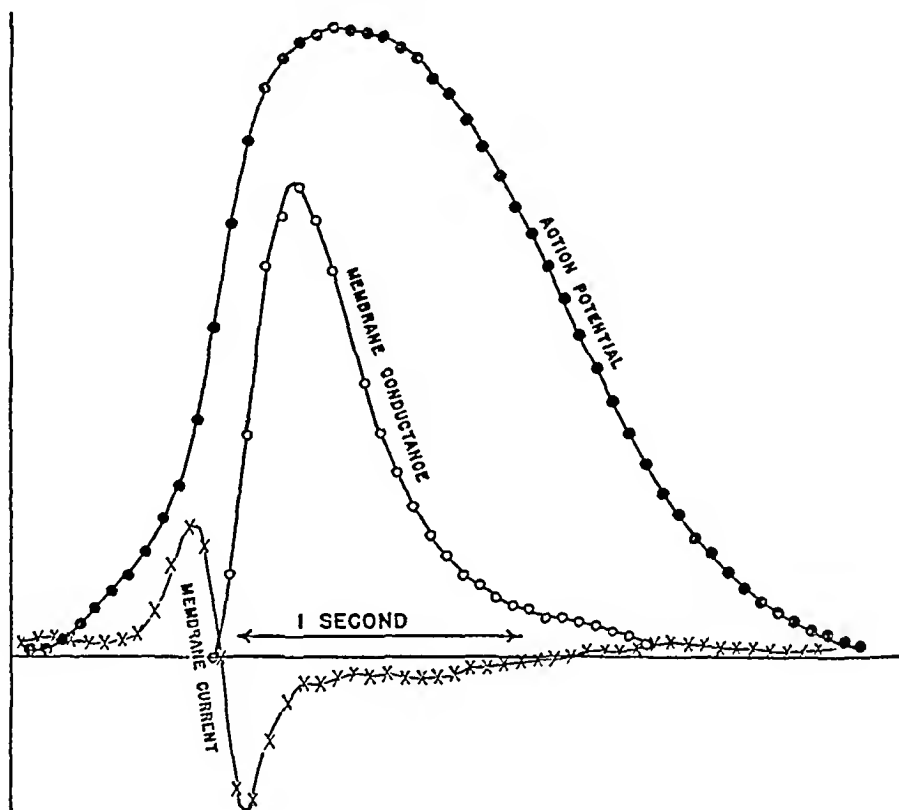


FIG 6 Membrane conductance, monophasic action potential, and membrane current *vs* time after stimulation Ordinates are all in arbitrary units Frequency 0.20 kc

the effective length was about 1.0 mm or twice the actual electrode length At a velocity of 1 cm/sec this would then correspond to a time of rise of 0.10 second and since 0.25 second or less is observed, the actual time is not more than 0.15 second Experiments in which the cell was stimulated under the impedance electrodes indicate that the time of rise may be considerably less than 0.15 second

If the flow of water through the cell was stopped the impedance

remained unchanged for fairly long periods of time unless the cell was stimulated. After a single stimulation, the low frequency impedance failed to return to its previous value but remained at a lower value for some time. On repeated stimulation the impedance became progressively lower, but there was an immediate return to the initial value when the water circulation was resumed. This indicates that the decrease of impedance was due to a decrease in the specific resistance of the medium which was probably due to a loss of electrolyte from the cell during excitation, that was recovered very slowly, if at all.

*Action Potentials*—It is seen in Fig 6 that the start of the membrane conductance change comes toward the maximum of the monophasic

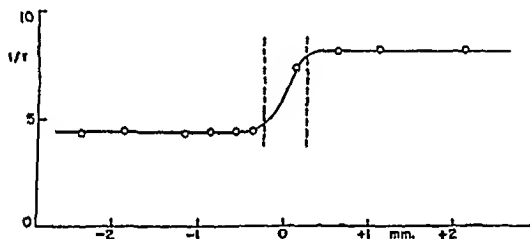


FIG 7 A glass rod in place of *Nitella* in measuring cell is moved from left to right. Conductance,  $1/r$ , vs distance of the end of the rod from the center of the electrodes. Negative abscissae indicate that the rod was between the electrodes. The dotted lines show the actual electrode width. Conductance is in arbitrary units.

action potential, and it is interesting to correlate it with the density of current flowing across the membrane. This is proportional to the second derivative of the action potential (equation (10)) and has been computed numerically to give the "membrane current" curve of Fig 6. The maximum values of the outward and inward current densities are approximately equal, although the low membrane resistance should allow a considerably greater maximum current density inward than outward. The impedance electrode width should tend to equalize these maxima but the effect has not yet been calculated or investigated with a model.

Not only are experiments with cells having one end in contact with  $M/10$  KCl not entirely satisfactory, and perhaps open to question on physiological grounds, but the cumulative errors in the numerical calculation of the second derivative led us to do a few experiments with a different technique

The *Nitella* cell in a moist chamber was supported on 2 platinized platinum wires, 0.25 mm in diameter, so as to make contacts on opposite sides of the cell, the wires serving as transverse impedance

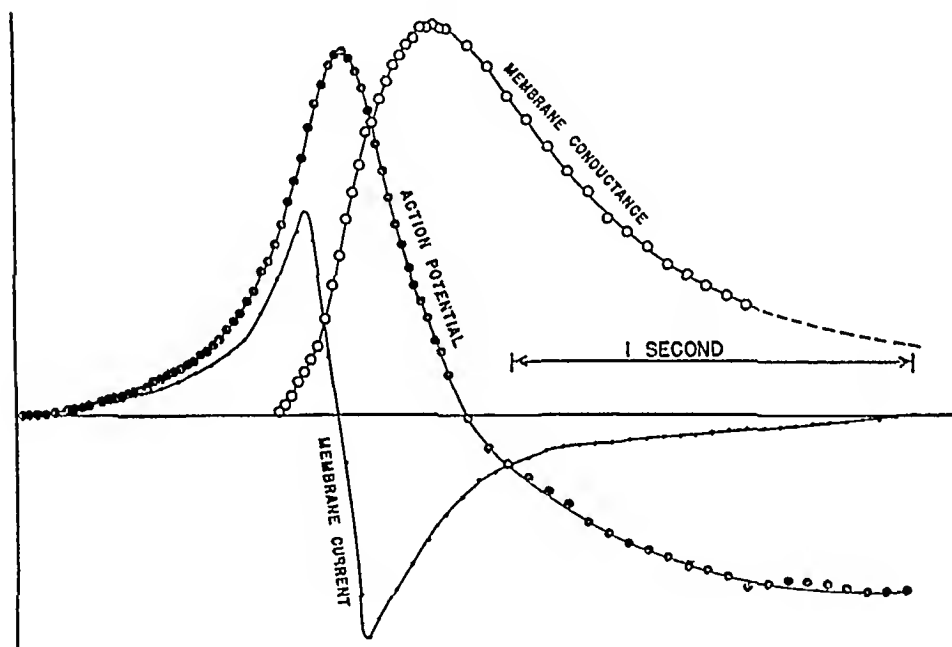


FIG 8 Membrane conductance, diphasic action potential, and membrane current measured in a moist chamber vs time after stimulation. Ordinates are all in arbitrary units. Frequency 0.20 kc

electrodes. On each side of one impedance electrode and 1 mm away from it a similar wire potential electrode touched the *Nitella* cell. These two were then differential electrodes giving the potential gradient over a 2 mm stretch which included the impedance electrodes. This "diphasic" potential was then approximately the first differential of the monophasic action potential, or proportional to the external current flow parallel to the long axis of the cell, and the membrane current density its first derivative.

The impedance and potential changes were taken at 64 frames per second and the results of an action are shown in Fig. 8. Although the resting characteristics are much less steady than in the flowing medium, and the results less reproducible, it is seen that the characteristics are entirely similar. It thus seems quite certain that in general the increase in conductance and the change in direction of the membrane current from outward to inward occur at very nearly the same time.

The typical action potential of Fig. 6, which has a form similar to that of nerve and a duration of 2 or 3 seconds, is quite different from the potentials with a double peak and a 15 second duration normally found by Osterhout (1934). We have obtained the latter type of monophasic action potential by keeping the cells in a medium similar to that used by Osterhout and although the relatively refractory period was longer and the impedance changes less reproducible, the results were essentially the same as those found for the more rapid cells.

#### DISCUSSION

*Impedance*—Although it is not yet possible to formulate our information on the electrical properties of a cell membrane in terms of a definite molecular structure, some characteristics seem fairly clear.

In Figs. 4 and 5, the extrapolated infinite frequency resistance is a common end point which is approached along one path for the resting cell as the frequency is increased and along another, at one frequency, during activity. The former path is most simply described as a decrease of the parallel reactance of the membrane and the latter as a decrease of its parallel resistance. Since also this parallel reactance corresponds to the membrane capacity found for a variety of other cells, a parallel capacity and resistance represent the membrane fairly well.

Observations on the excitability and impedance of the squid giant axon (Curtis and Cole, 1938) and the constancy of the red blood cell membrane capacity in chemical hemolysis (Fricke and Curtis, 1935) have led to the suggestion that the capacity and leakage resistance of the membrane might be relatively independent. In the present results, we have a sounder basis for this separation of the non con

ducting and conducting—or probably the ion impermeable, and ion permeable—aspects of the membrane

The change in the non-conducting aspect of the membrane is important in fertilization of marine eggs and may play a considerable rôle in the action of lipoid solvents, but it is apparently a minor factor in the excitation of *Nitella*, where the phase angle is unchanged and capacity is decreased by only 15 per cent. Of course, this capacity alteration may be very significant, but we shall pass over it for the present in view of the change in the conducting aspect of the membrane

Taking Blinks' (1930) value of  $10^5$  ohm  $\text{cm}^2$  for the resting *Nitella* membrane, we find a 200-fold increase in the membrane conductance at the height of activity. Yet the maximum conductance, corresponding to 500 ohm  $\text{cm}^2$  is far from a complete ionic permeability, which, if the resting resistance were not known, might seem to be a fairly high specific resistance when the large membrane capacity is taken as an indication of its thinness. The nearly intact capacity cannot be detected on Blinks' (1936) records because the active membrane time constant is less than  $10^{-3}$  second and the difference between his initial and final resistances is about 0.5 per cent of the resting value. However, it seems unreasonable to picture the depolarization process during activity as a destruction or disintegration of the membrane, and one may well suppose that no more than 15 per cent of the membrane is involved.

In many of the cells and tissues which have been measured, the cell membranes have been found to have an impedance of the form  $z_s = z_c(j\omega)^{-\alpha}$  (see page 56). This has been called a "polarization" impedance because this term has been applied to several types of non-living systems which have a similar but unexplained characteristic. Some of these systems are conductors and it was thought that a polarization impedance might be an expression of the ion permeability aspect of the membrane. Since a more easily acceptable measure of ion transport, *i.e.* the conductance, has been measured and its effect is probably so small as not to enter into the transverse impedance measurements of most resting cells and tissues, we are inclined to assume that the polarization impedance is essentially a dielectric impedance. The dielectric impedance is then to be

thought of as a characteristic of the ion impermeable aspect of the membrane and the constant  $\alpha$ , and the phase angle  $\phi$ , which is a measure of it ( $\phi = \alpha\pi/2$  radians), are measures of the dielectric loss, which occurs when  $\alpha < 1.0$ . This loss, which might be caused by a frictional resistance to dipole rotation, is expressed as an electrical resistance, but it is not to be confused with a resistance which is an expression of ion transport across the membrane. Some time after a change,  $\Delta V$ , of the potential difference across the membrane, there will be a change,  $\Delta I$ , in the membrane current density and by Ohm's law, the membrane resistance for unit area is given by  $r_4 = \Delta V/\Delta I$ . An ionic permeability of the membrane may then be defined as the number of ions transported across a unit area in unit time for a unit potential difference. The permeability on this definition is then proportional to  $\Delta I/\Delta V = 1/r_4$ , or the membrane conductance. There will also be accumulations of electrical charge on the two sides of the membrane,  $+\Delta Q$  and  $-\Delta Q$  and by the definition of capacity  $\Delta Q = c_m \Delta V$ , where  $c_m$  is the membrane capacity for a unit area (see Fig. 9). If now the ionic permeability be defined as the number of ions transported across the membrane in unit time for an ion pair separated by the membrane, this permeability is given by  $\Delta I/\Delta Q = 1/r_4 c_m$ , which is also the reciprocal of the time constant of the membrane. Then for resting *Nitella* we find a permeability of ten ions per second for one ion pair separated by the membrane and for the maximum permeability during excitation, two thousand ions per second for one ion pair.

*Action Potential*—The membrane polarization, which is made known by resting and action potentials, represents a source of electrical energy in the cell and it is preferable to speak of it and measure it, whenever possible, as an electromotive force (EMF) which is a physically defined quantity. The resting and action potentials are the externally measured potential differences due to the external current (equation (14) and Fig. 12), and are not necessarily synonymous with the membrane EMF. Consequently if the "depolarization" be considered as a decrease in the membrane EMF, it is not directly measured by the external potential difference, but differs from it as indicated by equation (15) on p. 61.

It has been pointed out that the decrease in membrane resistance,



which is a "loss of polarizability," does not occur until rather late in the rising phase of the action potential, and that the reversal of the membrane current flow takes place at nearly the same point. It has also been found that the foot of the curve of the action potential up to the time of the resistance change and current reversal is nearly exponential—as it should be in front of a resting or propagated localized membrane short circuit (see equation (17)). Furthermore, preliminary experiments on the membrane resistance and potential difference at the site of sub-threshold and threshold stimuli also indicate a close parallelism between the resistance and  $EMF$ . For

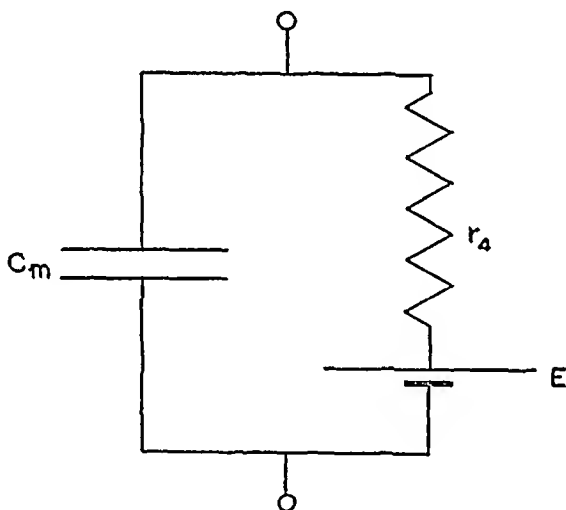


FIG 9 Equivalent membrane circuit  $C_m$ ,  $r_d$ , and  $E$  are respectively the capacity, resistance, and  $EMF$  of the membrane

these reasons, we shall assume that the membrane resistance and  $EMF$  are so intimately related that they should be considered as series elements in the hypothetical equivalent membrane circuit as shown in Fig 9. These two elements may be just different aspects of the same membrane mechanism and the resistance may be, in part or entirely, the internal resistance of the  $EMF$ .

On the basis of this circuit the usual cable differential equation (equation (12)), can be set up, following Cremer (1899), Hermann (1905), and others, to describe the behavior of the fiber at any point. If the membrane  $EMF$ ,  $E$ , is now considered as an unknown, we

should have all of the quantities necessary to calculate it. By assuming a resting membrane resistance ( $r_i$ , Fig. 9) somewhat lower than  $10^5$  ohm  $\text{cm}^2$  the membrane  $\Sigma MF$  remains nearly constant until the time of the resistance decrease, and it then follows the action potential quite closely. This is, however, merely another way of saying that the initial portion of the action potential is exponential, but to a certain extent it justifies the assumptions.

If then there is no evidence of membrane change in the initial portion of the action potential and the propagation depends upon such a change, the impulse cannot be said to have arrived. Although the conditions for the change in the  $\Sigma MF$  and conductance of the membrane may have been partially achieved, the phenomenon is, up to this point, entirely passive and is to be identified with the "electrotonic spread," as shown by Bogue and Rosenberg (1934), and others. An impulse cannot be said to have passed a point or to be blocked until the membrane  $\Sigma MF$  and resistance changes either have occurred or failed to take place.

It is apparent that the work to date has not shown whether excitation and propagation are possible with the assumed membrane and cell structure (*cf.* Rashevsky (1933), Rushton (1937)). This question can be answered as soon as we can set up the necessary and sufficient conditions for the  $\Sigma MF$  and resistance changes to occur. One can think of many threshold conditions for this change—a current density, a transport of ions, a change of membrane potential difference, a change of membrane  $\Sigma MF$ , resistance, or capacity. Without a good experimental indication of which one, or combination, of these and perhaps other conditions is most important, we do not feel prepared to go into a further analysis of the processes of excitation, conduction, and recovery.

### *Theory*

*Bridge Unbalance*—When a voltage  $e_i$  is applied to an equal ratio arm bridge having matched input and output impedances, it is found that near balance, the output voltage  $e_o$  is given by

$$e_o = \frac{1}{8} \frac{z - z'}{z + z'} e_i$$

where  $\bar{z}$  and  $z$  are the known and unknown impedances. Owing to the failure to meet all of these conditions and the attenuation, amplification, and phase shift of the input and output systems, we have in general

$$E_0 = \mu \frac{\bar{z} - z}{\bar{z} + z} E_1$$

where  $E_1$  and  $E_0$  are the oscillator and amplifier output voltages respectively, and  $\mu$  is the resultant complex amplification factor

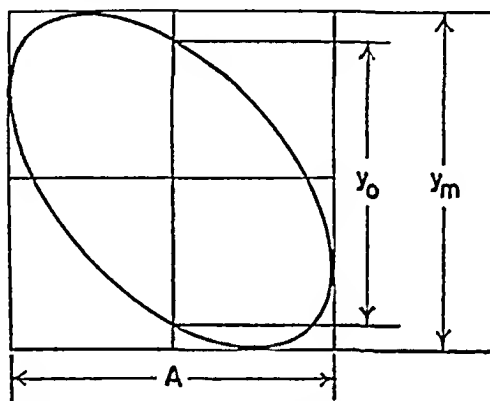


FIG 10 Ellipse to show the measurements taken to compute the impedance changes

Then

$$E_0 = \mu \bar{E}_1 \frac{\bar{z}z}{\bar{z} + z} \left[ \frac{\bar{R}_p - R_p}{\bar{R}_p R_p} \cos \omega t + \omega (\bar{C}_p - C_p) \sin \omega t \right]$$

where  $\bar{R}_p$ ,  $\bar{C}_p$  are the parallel resistance and capacity at balance,  $R_p$ ,  $C_p$  are the off-balance values, and  $\bar{E}_1$  is the amplitude of the oscillator voltage. This output voltage,  $E_0$ , gives a vertical deflection on the cathode ray oscillograph which may be written, approximately,

$$y = \frac{A}{2} (l_1 \Delta R_p \cos \omega t + l_2 \Delta C_p \sin \omega t) \quad (1)$$

where  $\Delta R_p$ ,  $\Delta C_p$  are the amounts by which the parallel resistance and capacity are off-balance, and  $A$ ,  $l_1$ , and  $l_2$  are obtained from the previous equation and the oscillograph sensitivity. In practice, the values of  $l_1$  and  $l_2$  are determined by direct calibration.

A portion of the oscillator output may be shifted in phase and amplitude so that it gives a horizontal deflection on the oscillograph

$$x = \frac{A}{2} \cos \omega t \quad (2)$$

We may then eliminate  $t$  between equations (1) and (2) and show that the oscillograph pattern is an ellipse which has a horizontal axis when  $\Delta R_p = 0$  and degenerates into a straight line inclined to the horizontal when  $\Delta C_p = 0$ .

When  $x = 0$ , we have  $y_0 = A l_2 \Delta C_p$  as shown in Fig. 10. On the other hand the maximum value of  $y$  is given by

$$y_m = A \sqrt{(l_1 \Delta R_p)^2 + (l_2 \Delta C_p)^2}$$

Thus we have

$$\left. \begin{aligned} \Delta C_p &= \frac{y_0}{A l_2} \\ \Delta R_p &= \frac{1}{A l_1} \sqrt{y_m^2 - y_0^2} \end{aligned} \right\} \quad (3)$$

which are the equations used for calculation.

*Transverse Impedance*—The theoretical background of the impedance of suspensions of spheres and cylinders has been developed in a series of papers (Cole, 1928, 1932, 1937, Cole and Curtis, 1936, Bozler and Cole, 1935, Curtis and Cole, 1937) but will be briefly outlined here to give a basis for the extensions which are now necessary.

When the alternating current flow is perpendicular to the axis of a parallel suspension of circular cylindrical cells or to the axis of a single cylindrical cell in a nearly square cross section of conducting medium, the specific transverse impedance,  $z$ , is given by

$$= r_1 \frac{(1 - \rho)r_1 + (1 + \rho)(r_2 + \pi/a)}{(1 + \rho)r_1 + (1 - \rho)(r_2 + \pi/a)} \quad (4)$$

where  $r_1$  and  $r_2$  are the specific resistances of the external medium and cell interior,  $z_m$  is the impedance for a unit area of membrane,  $a$  is the radius of the cell, and  $\rho$  is its volume concentration in the medium.

The overall or gross impedance,  $z$ , is represented by its component series resistance and reactance,  $z = r + jx$ , which may be plotted as abscissa and ordinate to give the impedance locus as it varies with frequency

Equation (4) has a well known form and many of its properties which are applicable here have been worked out in general terms (Campbell, 1911), but for simplicity a specific treatment will be followed

When  $z_m$  is either zero or infinite,  $z$  becomes a pure resistance,  $r_\infty$  or  $\bar{r}_0$ . For all physically realizable values of  $z_m$  all possible values

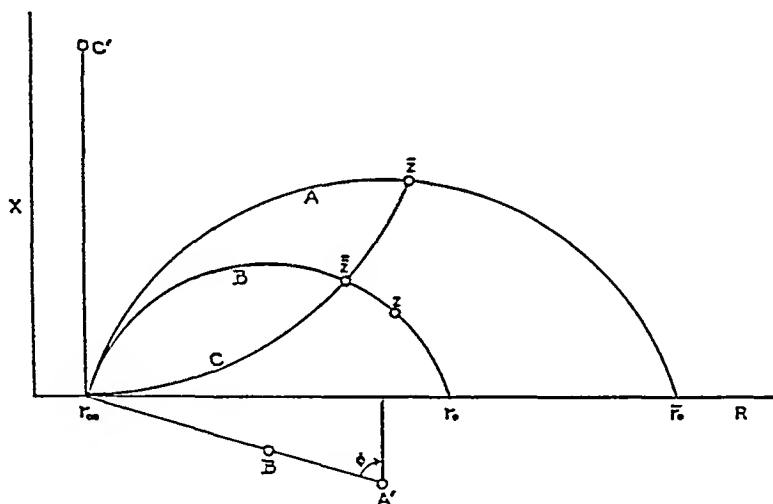


FIG 11 Theoretical impedance loci, showing loci due to change of frequency,  $A$  and  $B$ , with centers at  $A'$  and  $B'$  and the locus  $C$  due to a change of membrane resistance, with center at  $C'$

of  $z$  lie on or within a circle having the segment of the  $r$  axis between  $r_\infty$  and  $\bar{r}_0$  for a diameter

It is further found by simple rearrangement that

$$z_m = a \frac{\bar{r}_0^2 - r_1^2}{\bar{r}_0 - r_\infty} \frac{z - r_\infty}{\bar{r}_0 - z} \quad (5)$$

This is a general expression which is convenient to use in special cases

In many biological materials,  $z_m$  is a polarization or dielectric impedance,  $z_m = z_3 = z_c (j\omega)^{-\alpha}$ , where  $z_c$  is a constant,  $j = \sqrt{-1}$ , and  $\omega$  is  $2\pi$  times the frequency, and the impedance locus, as  $\bar{z}$  or  $\omega$  is varied, is a circular arc from  $r_\infty$  to  $\bar{r}_0$  which subtends the angle

$2\phi = \alpha\pi$  at its center. When the membrane has a parallel leakage resistance,  $r_4$ , which is independent of frequency,  $1/z_m \approx 1/z_3 + 1/r_4$ , the value of  $r_\infty$  is unchanged but when  $z_3$  is infinite,  $z_m = r_4$  and  $\bar{r}_0$  is decreased to another value  $r_0$ . Then as  $\bar{z}$  or  $\omega$  vary the impedance locus is a circular arc of angle  $2\phi$  from  $r_\infty$  to  $r_0$ , and the value  $r_4$  may be calculated from equation (5) or

$$r_4 = a \frac{r_0^2 - r_\infty^2}{r_0 - r_\infty} \frac{r_0 - r_\infty}{\bar{r}_0 - r_0} \quad (6)$$

Measurements at zero frequency where  $z = r_0$  or  $\bar{r}_0$  cannot verify the assumption that  $r_4$  is independent of frequency, but when it can be shown that  $\phi$  and  $r_\infty$  are unchanged, the value of  $r_0$  corresponding to any value of  $z$  can be extrapolated graphically by a circular arc of angle  $2\phi$  through  $r_\infty$  and  $z$ . It is, however, possible to find the path which is followed by  $z$  when  $r_4$  changes but  $z_3$  and  $r_\infty$  remain constant, which is the membrane resistance locus at constant frequency and membrane capacity. If  $z$  is the value of  $z$  for some value of  $z_3$  when  $r_4$  is infinite, then equation (4) becomes

$$z = \frac{\sigma r_\infty + z r_4/a}{\sigma + r_4/a} \quad (7)$$

where

$$\sigma = \frac{\bar{r}_0^2 - r_\infty^2}{(r_0 - r_\infty)^2} (r_0 - r_\infty)$$

It is known that equation (7) is again a circle and that it passes through the points  $r_\infty$  and  $z$  but its center and radius must be found. Separating the real and imaginary parts, we have the two equations,

$$(r - r)r_4/a = \bar{r}_0^2[(r - r_\infty)(r - r_\infty) - x\bar{x}]/(r_0 - r_\infty)^2$$

$$(x - x)r_4/a = \bar{r}_0^2[(r - r_\infty)x + (r - r_\infty)\bar{x}]/(r_0 - r_\infty)^2$$

where  $r$ ,  $x$  and  $\bar{r}$ ,  $\bar{x}$  are the components of  $z$  and  $\bar{z}$ . By eliminating  $r_4$  we find

$$r^2 + x^2 - 2r_\infty r + [(r - r_\infty)^2 + x\bar{x}]/\bar{x} + r_\infty^2 = 0 \quad (8)$$

which is the equation of a circle having its center at the point  $r_\infty$ ,  $(\bar{r}^2 + \bar{x}^2)/2\bar{x}$  and the radius  $(\bar{r}^2 + \bar{x}^2)/2\bar{x}$ . This circular locus then passes through  $\bar{z}$  and is tangent to the resistance axis at  $r_\infty$  as shown in Fig. 11.

When at constant frequency,  $z$  does not follow the locus required by a variation of  $r_1$  only,  $z_3$  must vary also, and when it can be shown that  $\phi$  does not change, we need only the absolute value of the polarization or dielectric impedance  $|z_3|$ . From equation (5) we find that when  $r_1$  is infinite

$$|\bar{z}_3| = a \frac{\bar{r}_0^2 - r_1^2}{\bar{r}_0 - r_\infty} \frac{\bar{u}}{\bar{v}}$$

where  $\bar{u}$  and  $\bar{v}$  are the lengths of the chords to  $\bar{z}$  from  $r_\infty$  and  $\bar{r}_0$  respectively. When  $r_1$  is finite,

$$|z_3| = a \frac{\bar{r}_0^2 - r_1^2}{\bar{r}_0 - r_\infty} \frac{r_0 - r_\infty}{\bar{r}_0 - r_\infty} \frac{u}{v}$$

where  $u$  and  $v$  are the chords to  $z$  from  $r_\infty$  and  $r_0$ . But if the point  $\bar{z}$  is on the resistance locus as shown in Fig. 11, then we know that  $|z_3|$  has the value  $|\bar{z}_3|$  at this point so

$$|\bar{z}_3| = a \frac{\bar{r}_0^2 - r_1^2}{\bar{r}_0 - r_\infty} \frac{r_0 - r_\infty}{\bar{r}_0 - r_\infty} \frac{\bar{u}}{\bar{v}}$$

where  $\bar{u}$  and  $\bar{v}$  are the chords to  $\bar{z}$  from  $r_\infty$  and  $r_0$ . We then have

$$|z_3|/|\bar{z}_3| = \frac{u}{\bar{v}} \bigg/ \frac{\bar{u}}{\bar{v}}$$

The membrane capacity,  $c = 1/|z_3| \omega \sin \phi$  and

$$c/\bar{c} = \frac{\bar{u}}{\bar{v}} \bigg/ \frac{u}{v} \quad (9)$$

There are other useful methods for the calculation of  $z_3$  and  $r_1$ , as well as variations of these given and approximations which may be used judiciously.

*Cable Theory*—A nerve fiber has been treated as a succession of circuit elements by Hermann (1905) and as the elements approach zero length this becomes the uniform cable which was investigated by Cremer (1899) and others, following Kelvin (1856). Although the results of the theory have been applied to whole nerves, the postulates should be much better represented by *Nitella*. There can be little question that the resting fiber has properties which

are described by such an equation and the equations are developed here for purposes of consolidation and consistency of nomenclature.

Let  $r_1$  and  $r$  be the resistance for a unit length of the medium, and the cell interior respectively,  $z_m$  the impedance between inside and outside for a unit length. Let  $i_1$ ,  $i_2$ , and  $i_m$  be the external, internal, and membrane currents in the positive direction, and  $V_1$  and  $V_2$  be

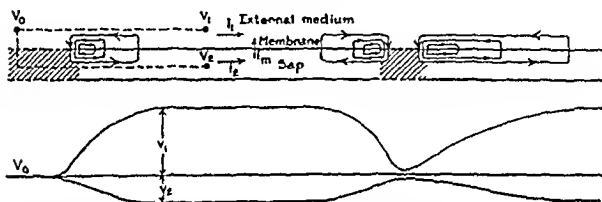


FIG. 12. Schematic drawing of *Nitelloid* cell to show currents and potentials in the neighborhood of a depolarized region and an inactive end at the left.  $i_1$ ,  $i_2$ , and  $i_m$  are the external, internal, and membrane currents (for direction of arrows see p. 59) and the other arrows in the figure indicate the local circuit current flow.  $V_0$  is the reference potential and  $V_1$  and  $V_2$  are the external and internal potentials.

the potentials outside and inside, as shown in Fig. 12. Then by Ohm's law

$$\frac{\partial V_1}{\partial x} = -r_1 i_1, \quad \frac{\partial V_2}{\partial x} = -r_2 i_2, \quad V_1 - V_2 = -z_m i_m$$

When there is no current flow to and from the system considered

$$i_1 + i_2 = 0 \quad \text{and} \quad \frac{\partial i_1}{\partial x} = -\frac{\partial i_2}{\partial x} = i_m$$

Then

$$\frac{\partial^2 V}{\partial x^2} = -(r_1 + r_2) i_m \quad (10)$$

where  $V_1 - V_2 = V$

For the reasons given above, we shall assume an equivalent membrane circuit given in Fig. 9. The dielectric impedance of the mem-



brane has been found to have a phase angle of about  $80^\circ$ , but for an approximate analysis we shall replace it by the pure capacity  $c_m$  with a  $90^\circ$  phase angle

Let

$$z_3 = -c_m \frac{\partial V}{\partial t}, \quad z_4 = \frac{E - V}{r_4} \quad (11)$$

and

$$z_m = z_3 + z_4 = -c_m \frac{\partial V}{\partial t} + \frac{E - V}{r_4}$$

Substituting

$$\frac{\partial^2 V}{\partial x^2} = - \frac{r_1 + r_2}{r_4} \left[ E - V - r_4 c_m \frac{\partial V}{\partial t} \right] \quad (12)$$

and when we let  $\lambda = \sqrt{r_4 / (r_1 + r_2)}$ , the characteristic length, and  $\tau_m = r_4 c_m$ , the membrane time constant,

$$\lambda^2 \frac{\partial^2 V}{\partial x^2} - \tau_m \frac{\partial V}{\partial t} - V = -E \quad (13)$$

This is now the general equation relating all of the assumed characteristics of the cell as functions of space and time

But  $V$  cannot be easily measured directly and the usual approach is by the inactive end technique. Choosing an electrode at  $x = 0$  on the inactive end as the point of zero potential for  $V_1$  and  $V_2$ , then the externally measured potential  $V_1$  at an uninjured point  $x$  is given by

$$V_1(x) = \int_0^x \frac{\partial V_1}{\partial x} dx = -r_1 \int_0^x i_1 dx \quad (14)$$

In other words, the resting or injury potential is caused entirely by local current flow at the junction of the injured and uninjured regions. Assuming that the injury is of such a kind that the membrane potential is abolished, then there is no potential difference across the membrane at  $x = 0$ , and the potential inside the fiber  $V_2$  at  $x$  is

$$V_2(x) = \int_0^x \frac{\partial V_2}{\partial x} dx = -r_2 \int_0^x i_2 dx = -\frac{r_2}{r_1} V_1(x),$$

since  $i_1 + i_2 = 0$

Thus

$$I = V_1(x) - I_1(x) = \left(1 + \frac{r_1}{r_2}\right) I_1(x) = \kappa V_1(x) \quad (15)$$

We may now define a reduced  $E_1$  by  $E = \kappa E_1$  and drop the subscripts to obtain the same equations, where  $V$  is now the measured "monophasic" potential

Once the impulse has been initiated and is traveling with a uniform velocity,  $v$ , we may refer it to a space or time coordinate system whose origin travels with the same velocity. Since the length of an impulse is of the order of a centimeter for velocities from a centimeter to a hundred meters per second, a space coordinate is to be preferred where  $y = x - vt$

Then

$$\frac{\partial V}{\partial x} = \frac{\partial I}{\partial y} \quad \frac{\partial^2 V}{\partial x^2} = \frac{\partial^2 V}{\partial y^2} \quad \text{and} \quad \frac{\partial I}{\partial t} = -v \frac{dV}{dy}$$

and we have

$$\lambda^2 \frac{d^2 V}{dy^2} + v r_m \frac{dV}{dy} - V = -E$$

which may be rewritten for calculation,

$$E = V - \beta \lambda^2 \left[ \frac{d^2 V}{dy^2} + \frac{1}{\lambda_0} \frac{dV}{dy} \right] \quad (16)$$

where  $\beta = r_1/\bar{r}_1$ ,  $\bar{r}_1$  is the membrane resistance at rest, and  $\lambda_0 = 1/v(r_1 + r_2)c_m$ , the characteristic length for a non conducting membrane.  $\tilde{\lambda} = \sqrt{\bar{r}_1/(r_1 + r_2)}$  is the characteristic length due to membrane conductance while  $\lambda_0$  is the characteristic length due to the membrane capacity

When we propagate a localized short circuit, so that  $E$  and  $r_1$  have their resting values at all points except  $y = 0$ , where they are both zero, and  $V = E - c$

$$\lambda^2 \frac{d^2 c}{dy^2} + v r_m \frac{dc}{dy} - c = 0$$

Since  $\tilde{\lambda}$  and  $\lambda_0$  are constant except at  $y = 0$ ,

$$c = c_0 \exp(-y/\lambda_0) \quad (17)$$

Thus outside of the breakdown the action potential should be of exponential form with a characteristic length

$$\lambda_a = \frac{\bar{\lambda}}{\frac{v\tau_m}{2\bar{\lambda}} \pm \sqrt{1 + \frac{v^2\tau_m^2}{4\bar{\lambda}^2}}}$$

using the positive sign for  $y > 0$  which is ahead of the short circuit and the negative sign for  $y < 0$  which is behind it. If the velocity

$v$  is small as for *Nitella*, then  $\lambda \gg \lambda_0$  and  $\lambda_a = \lambda \pm \frac{v\tau_m}{2}$ , approaching

$\bar{\lambda}$ , the resting characteristic length. For a high velocity  $\bar{\lambda} \ll v\tau_m$ ,  $\lambda_a$  ahead approaches  $\lambda_0$ , which is independent of  $r_4$ , but behind becomes  $v\tau_m$ .

#### CONCLUSION

The transverse impedance measurements over a range of frequencies indicate that the impedance changes observed during activity are due entirely to changes in the membrane characteristics. By an extension of the transverse impedance theory it can be shown that, while there is no measurable change in the membrane phase angle (see Figs 4 and 5), the membrane capacity decreases at the height of activity about 15 per cent below its resting value of  $0.9 \mu\text{f}/\text{cm}^2$ . As compared with this, the change of the membrane resistance from a probable value of  $10^6 \text{ ohm cm}^2$  to an average value of  $500 \text{ ohm cm}^2$  at the height of activity is very great (see Figs 6 and 8). This is taken as additional evidence that the capacity represents the non-conducting aspect of the membrane and that the phase angle difference from  $90^\circ$  is a measure of the dielectric loss of the membrane, while the parallel membrane resistance is the conducting or ion permeable aspect. In the processes of excitation, conduction, and recovery, these two aspects seem to be quite independent of each other, and the non-conducting aspect is very little affected.

It has been found that the membrane conductance increases very rapidly late in the rising phase of the action potential at nearly the same time that the direction of the current flow across the membrane changes from outward to inward (see Figs 6 and 8). For this reason, we assume that the membrane  $\text{EMF}$  and resistance are closely related, the latter being perhaps the internal resistance of the  $\text{EMF}$ .

This structure is then considered to be in parallel with the relatively inactive membrane capacity. On this basis the membrane  $EMF$  is found to decrease sharply at nearly the time the conductance increases. It is then this sudden change of the  $EMF$  and conductance which is the basis of the all or none nature of the propagated activity. Although the conditions for this change may be partially satisfied before it occurs, we must consider the properties to be those of a resting cell and the part of the action potential ahead of the change to be a purely passive propagation. With these definite measurements of the time, course, and nature of the membrane changes and with an extension of the present technique, it should be possible to establish the necessary and sufficient conditions for the change, and so be a step closer to the understanding of the all-or none law.

#### SUMMARY

The changes in the alternating current impedance which occur during activity of cells of the fresh water plant *Nitella* have been measured with the current flow normal to the cell axis, at eight frequencies from 0.05 to 20 kilocycles per second, and with simultaneous records of the action potential under the impedance electrodes. At each frequency the resting cell was balanced in a Wheatstone bridge with a cathode ray oscillograph, and after electrical stimulation at one end of the cell, the changes in the complex impedance were determined from the bridge unbalance recorded by motion pictures of the oscillograph figure. An extension of the previous technique of interpretation of the transverse impedance shows that the normal membrane capacity of  $0.9 \mu f/cm^2$  decreases about 15 per cent without change of phase angle, while the membrane resistance decreases from  $10^5 ohm cm^2$  to about  $500 ohm cm^2$  during the passage of the excitation wave. This membrane change occurs during the latter part of the rising phase of the action potential, and it is shown that the membrane electromotive force remains unchanged until nearly the same time. The part of the action potential preceding these membrane changes is probably a passive fall of potential ahead of a partial short circuit.

We are very much indebted to Mr. J. M. Spencer for his assistance in this work.

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# KINETICS OF THE FORMATION OF PEPSIN FROM SWINE PEPSINOGEN AND IDENTIFICATION OF AN INTERMEDIATE COMPOUND

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Swine pepsinogen, the inactive precursor of swine pepsin, has recently been isolated from gastric mucosae and crystallized (1). This protein has no proteolytic or milk clotting action at pH 5.0-6.0 and is unchanged in solution at pH 8.5. When a solution of pepsinogen is made more acid than pH 5.0 it changes into the enzyme, pepsin. Pepsin, however, clots milk at pH 5.0-6.0 and is instantly inactivated at pH 8.5. Measurement of the milk clotting activity at pH 5.0-6.0, therefore, determines pepsin alone. Complete conversion of pepsinogen to pepsin at pH 2.0 followed by measurement at pH 5.0-6.0 determines pepsin plus pepsinogen, while pepsinogen alone may be estimated by inactivating the pepsin at pH 8.5 and then converting the pepsinogen at pH 2.0, followed by an estimation of the pepsin thus formed.

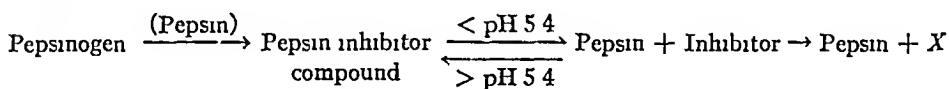
In the previous work (1) other methods of estimation were used to examine the formation of pepsin from crystalline pepsinogen at pH 4.6 and it was found that the reaction was autocatalytic, i.e., a product of the reaction, pepsin, accelerated the formation of itself. It was found that this transformation at pH 4.6 could be quantitatively described by a simple autocatalytic equation which states that the rate of reaction at any time is proportional to both the pepsin and pepsinogen concentration. At pH 4.6 the decrease in pepsinogen concentration was accompanied by a concomitant and equal increase in the pepsin.

In the present kinetic study the previous results at pH 4.6 have been confirmed and it has been found that under all conditions of varied acidity and salt the formation of pepsin from pepsinogen is

essentially autocatalytic for addition of pepsin always accelerates the reaction. However, in solutions more acid than pH 4.0 the loss in pepsinogen was not immediately accompanied by an equivalent increase in pepsin. Since the amount of pepsin ultimately reached the value equivalent to the total original pepsinogen concentration and for other reasons it is concluded that this discrepancy is not due to a secondary reaction involving either the pepsin or pepsinogen. The experimental facts agree with the assumption that in the formation of pepsin from pepsinogen there is an intermediate compound. The presence of this intermediate compound is responsible for the deviations from the simple autocatalytic kinetics. The compound differs from pepsinogen in being alkali (pH 8.5) labile like pepsin but differs from pepsin in being enzymatically inactive. The compound (*PI*) is a reversibly dissociable compound of pepsin (*P*) and a low molecular weight inhibitor (*I*), the dissociation being nearly complete below pH 5.0 and very small at pH 6.0.

The inhibitor is a peptide which is slowly destroyed in the presence of pepsin. It is soluble and stable in boiling 2.5 per cent trichloroacetic acid, insoluble in 0.7 saturated ammonium sulfate containing 2.5 per cent trichloroacetic acid, and dialyzes only slowly through collodion.

The transformation reaction may be schematically represented as follows:



The pepsin inhibitor and pepsin inhibitor compound have many properties in common with the trypsin inhibitor and trypsin inhibitor compound reported by Kunitz and Northrop (2). However, the trypsin inhibitor is not formed from trypsinogen upon conversion into trypsin but is a material occurring in the pancreas.

### *Experimental Results*

The experiments reported here present a general survey of the kinetics of the transformation of pepsinogen into pepsin. The general forms of the curves under the various conditions are significant and have been obtained repeatedly with various preparations but quantitatively there is considerable variation, owing to the fact that the

reaction rate is very sensitive to slight changes in pH, salt concentration, temperature, and pepsin or pepsinogen concentration

*Effect of Variation in Pepsin and Pepsinogen Concentration on the Reaction*

It was shown in the writer's previous work (1) that transformation of pepsinogen to pepsin at pH 4.6 followed the simple autocatalytic equation

$$-\frac{d(\text{pepsinogen})}{dt} = +\frac{d(\text{pepsin})}{dt} = K(\text{pepsinogen})(\text{pepsin})$$

or on integration

$$K = \frac{2.3}{(\text{pepsinogen}_{(0)})^2} \log_{10} \left[ \frac{(\text{pepsinogen}_{(t)})}{(\text{pepsinogen}_{(0)}) - (\text{pepsinogen}_{(t)})} \right] + C$$

where pepsinogen<sub>(0)</sub> is the concentration of pepsinogen at time = 0, pepsinogen<sub>(t)</sub> is the concentration of pepsinogen at time = *t*, and *C* the integration constant is equal to the logarithm of the ratio of pepsinogen concentration at time = 0 to the pepsin concentration at time = 0. This equation predicts that increasing the concentration of either pepsin or pepsinogen will result in an increase in rate of change in pepsinogen concentration per unit of time.

#### EXPERIMENTAL PROCEDURE

*A* To 3.4 ml water was added 0.4 ml 0.1 *N* pH 4.5 acetate buffer and 0.2 ml of a pepsinogen solution containing 2 mg protein nitrogen per ml. The solution was kept at 35.5°C. At intervals of time 0.5 ml samples were removed and the pepsinogen content determined as described under Experimental methods.

In a second tube 0.5 ml of a solution of crystalline pepsin prepared from Parke Davis pepsin at pH 4.5 containing 1 mg protein nitrogen per ml was added to 2.9 ml water followed by the acetate buffer and pepsinogen and treated in a similar manner.

*B* The same procedures and solutions were used in this experiment as in *A* with the exception that the temperature was 0°C and the buffer instead of pH 4.5 acetate was 0.1 *N* acetic acid. Pipettes and solutions were all cooled to 5°C before using.

*C* To 4 ml of 5 *N* hydrochloric acid was added 15.6 ml water at 0°C and 0.4 ml of pepsinogen containing 5 mg protein nitrogen per ml. The tube containing the solution was kept in cracked ice and at intervals of time 1 ml samples were removed with previously chilled pipettes and the pepsinogen estimated in the



usual way. In the alkaline borate solution used in the estimation of pepsinogen an equivalent of alkali was present in the borate solution and this solution was chilled to 0°C before introduction of the acid in order to minimize the effect of the strong alkali on the protein. In the second tube was 13.6 ml. water, 2 ml. of 1 mg protein nitrogen per ml. crystalline pepsin solution, 4 ml. 5 N hydrochloric acid and 0.4 ml. pepsinogen. This was treated in a manner analogous to that used on the solution in the first tube.

In Fig. 1 are the results of experiments in which the effect of adding pepsin to pepsinogen solutions was examined at pH 4.6, pH 3.0, and pH 0.

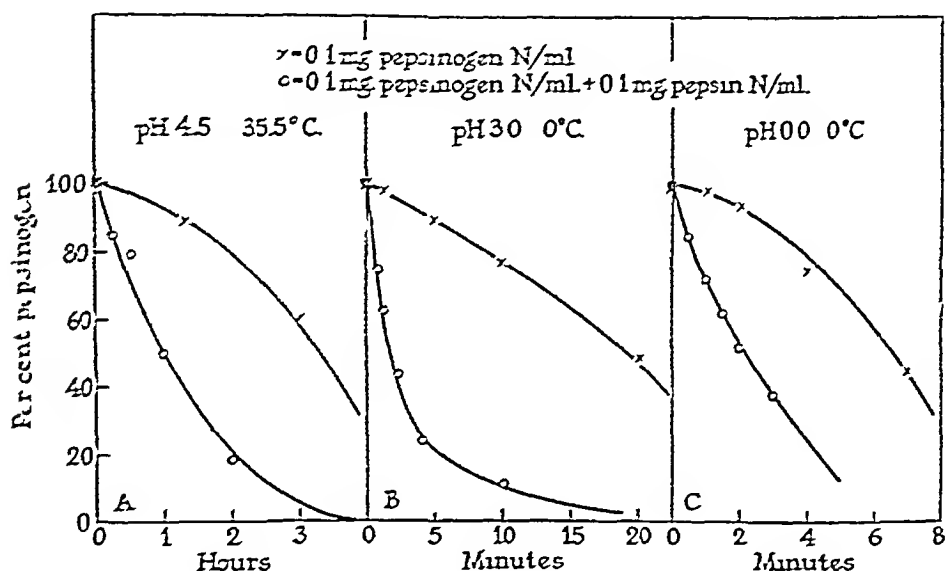


FIG. 1. Effect of pepsin on the transformation of pepsinogen at different pH.

and pH 0. In each instance the addition of pepsin caused an increase in the rate of reaction as measured by the loss in the pepsinogen. This result is in agreement with the hypothesis that the system is essentially autocatalytic in nature at all pH.

#### EXPERIMENTAL PROCEDURE

A. To 7.65 ml. water, 1 ml. M/1 pH 4.65 acetate buffer, and 0.35 ml. N/10 hydrochloric acid was added 1 ml. of 10 mg protein nitrogen per ml. pepsinogen solution. The mixture was kept at 35.5°C and at intervals samples were removed and analyzed for the amount of pepsinogen as described in Experimental methods.

Immediately after mixing the above reaction mixture 1 ml. was removed and added to 7.65 ml. water, 0.35 ml. N/10 sodium chloride, and 1 ml. M/1 pH 4.65

acetate all at 35.5°C. This solution now contains 0.1 mg protein nitrogen per ml. Aliquots were removed from time to time and analyzed for pepsinogen.

As soon as the above solution containing 0.1 mg protein nitrogen per ml was mixed 1 ml was removed and added to 7.65 ml water, 0.35 ml N/10 sodium chloride, and 1 ml M/1 pH 4.65 acetate and handled just as in the above instance. This solution contains 0.01 mg protein nitrogen per ml.

*B* The three solutions in *B* containing respectively 1.0, 0.1 and 0.01 mg protein nitrogen per ml were made up as described below and kept at 0°C. Samples were analyzed for pepsinogen.

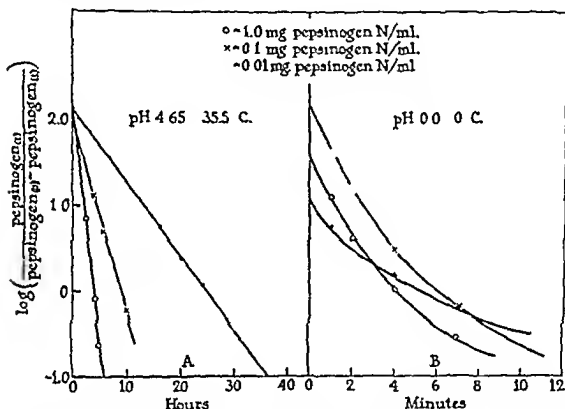


FIG. 2. Effect of pepsinogen concentration on the rate of transformation of pepsinogen at different pH.

"1.0"—4 ml 5 mg protein nitrogen per ml pepsinogen was added to 4 ml 5 N hydrochloric acid + 0.3 ml N/1 hydrochloric acid + 11.7 ml water all at 0°C.

"0.1"—4 ml of 0.5 mg protein nitrogen per ml pepsinogen + 4 ml 5 N hydrochloric acid + 12 ml water.

"0.01"—0.4 ml of 0.5 mg protein nitrogen per ml pepsinogen + 4 ml 5 N hydrochloric acid + 15.6 ml water.

In Fig. 2 are the results of experiments in which the pepsinogen concentration was varied at pH 4.6 and pH 0. At pH 4.6 an increase in pepsinogen concentration always resulted in an increase in the rate of reaction as measured by the loss in pepsinogen. Further

proof of the autocatalytic nature of the reaction is shown by the fact that the determined values fall on a straight line when the  $\log_{10} \left( \frac{\text{pepsinogen}_{(t)}}{\text{pepsinogen}_{(0)} - \text{pepsinogen}_{(t)}} \right)$  is plotted against the time, where pepsinogen  $_{(t)}$  is the concentration of pepsinogen at time  $t$  and pepsinogen  $_{(0)}$  is the original pepsinogen concentration

At pH 0 the results are not so clear. The reaction is more complicated since increasing the pepsinogen concentration does not cause a marked increase in rate of transformation and the reaction is not exactly linear when plotted as  $\log_{10} \left( \frac{\text{pepsinogen}_{(t)}}{\text{pepsinogen}_{(0)} - \text{pepsinogen}_{(t)}} \right)$  but proceeds more slowly as the reaction continues. There are a

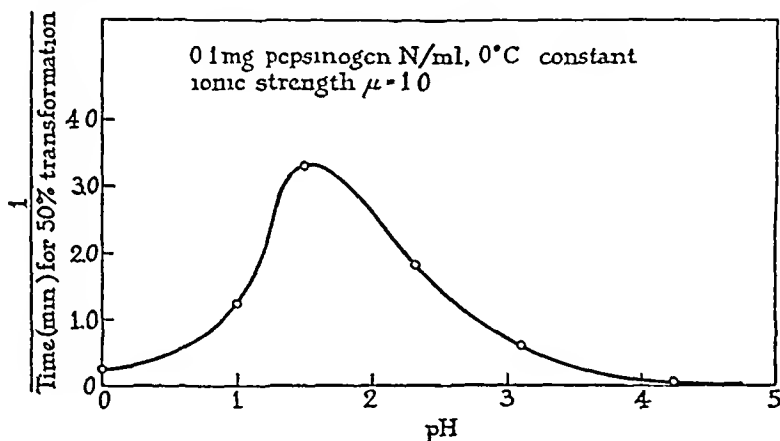


FIG 3 pH-pepsinogen transformation curve

number of explanations for these deviations but unfortunately experimental difficulties prevent a decisive answer on this point. However, since pepsin increases the rate of conversion at this pH it is highly probable that the reaction is still autocatalytic in nature but complicated for reasons to be described later in this paper.

#### *Effect of pH on the Rate of Transformation*

If the transformation is autocatalytic one would expect a maximum rate in the region of acidity where pepsin is most active or efficient, i.e., about pH 2.0. Since salts were known to affect the reaction it was necessary to have a constant ionic strength. The results of the experiments carried out in the presence of molar chloride ion at 0°C are shown in Fig 3 in which the reciprocal of the time necessary

for 50 per cent conversion is plotted against the pH. The curve is qualitatively similar to the pH digestion curve of pepsin (3) and is therefore a further indication that the reaction is catalyzed by pepsin.

#### EXPERIMENTAL PROCEDURE

All the solutions used in this experiment were  $M/10$  with respect to acetate ion and  $M/1$  with respect to chloride ion. The pH was determined electrometrically by a glass electrode standardized against  $M/10$  pH 4.0 acetate buffer. The solutions were kept in the ice bath during the entire experiment and chilled pipettes were used throughout. The volume, 5 ml, and pepsinogen concentration of 0.1 mg protein nitrogen per ml was the same in all tubes. The tubes differed only in the amount of hydrogen ion which was regulated by acetate buffer and hydrochloric acid. The pepsinogen was a heat denatured and reversed preparation which had subsequently been fractionated and dialyzed.

Changing the pepsinogen concentration changes the rate of reaction more on the alkaline side of the maximum than on the acid side. The shape of the pH rate of reaction curve will, therefore, vary somewhat with the pepsinogen concentration.

#### *Effect of Salt on Reaction*

If the change in pepsinogen catalyzed by pepsin is similar to the ordinary digestion of proteins by pepsin it would be expected that the rate of transformation would be depressed by the addition of neutral salts. It was found, however, as may be seen in Fig. 4A that the rate of reaction as measured by the loss in pepsinogen is increased by the addition of salts at pH 4.0. The pH of the solution was shifted as much as a half a pH unit but after correcting for this effect there is still a very definite accelerating effect. The change in pepsinogen still follows the autocatalytic equation as may be seen from the type of plotting in Fig. 4A. The addition of salt on the acid side of the pH transformation maximum has the opposite effect, as seen in Fig. 4B. Here the pH is also affected by the addition of salts but even correcting for this the salt depresses the rate of reaction. It might be pointed out that these experiments in Fig. 4B follow the autocatalytic plot more nearly than those at pH 0 in Fig. 2B.

#### EXPERIMENTAL PROCEDURE

The pepsinogen content of the solutions in both A and B was 0.1 mg protein nitrogen per ml and the temperature was 25°C. and 0°C. respectively. Aliquots

were removed from time to time and pepsinogen content determined as described under Experimental methods. The pH was determined electrometrically with a hydrogen electrode standardized against  $M/10$  pH 4.0 acetate buffer and  $M/10$  hydrochloric acid.

A In the presence of no salt  $M/10$  pH 4.0 acetate was used. With sodium chloride and magnesium sulfate pH 4.45 acetate was used and the resulting pH is recorded on Fig. 4A.

B The concentration of hydrochloric acid and sodium chloride and the E.M.F. of the activation mixture are indicated on Fig. 4B.

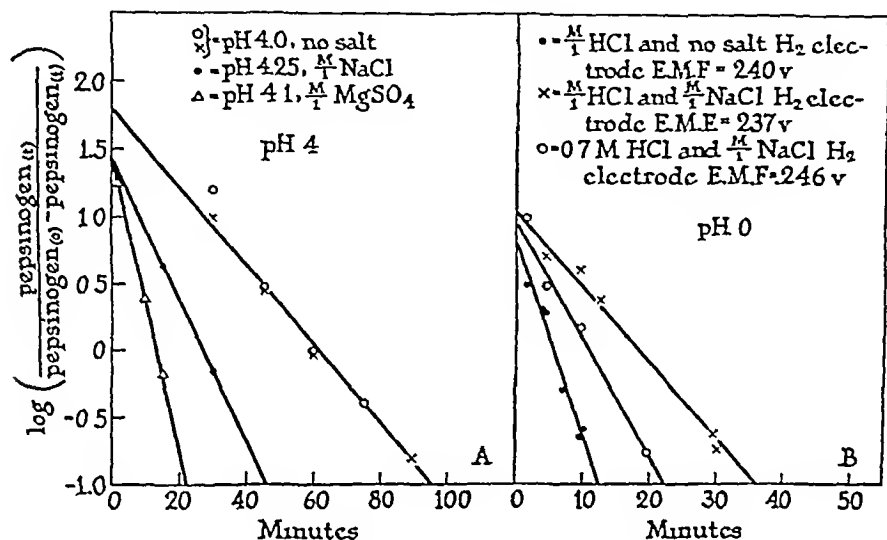


FIG. 4 Effect of salts on the rate of transformation of pepsinogen at different pH

#### *Evidence for an Intermediate Compound, (PI)*

As pointed out earlier in this paper, the decrease in pepsinogen should be equal to the increase in pepsin if the reaction consists simply of the conversion of pepsinogen to pepsin. This was the case in the experiments first reported (1) which were carried out at pH 4.6. However, when similar experiments were carried out in solutions more acid than pH 4.0 it was found that the decrease in pepsinogen was faster than the increase in pepsin. This is shown clearly in Fig. 5 where the changes in pepsinogen and pepsin were examined at pH 4.6, pH 4.0, pH 0, and at pH 4.0 in the presence of molar magnesium sulfate. It may be seen that at pH 4.6 the amount of pepsin found is equal at any time to the amount of pepsinogen lost, thus confirming the previous work. Under the other conditions, however, when

the pepsinogen was completely altered there was only a fraction of the pepsin present. On longer standing the pepsin concentration always reached a value equivalent to the total original pepsinogen concentration. The discrepancy between the pepsinogen lost and the pepsin found cannot be due to the irreversible destruction of pepsin nor to any secondary reaction involving either pepsin or pepsinogen.

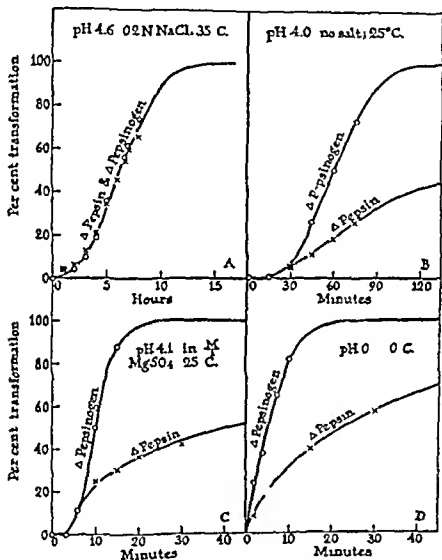


FIG. 5. Comparison of increase in pepsin with decrease in pepsinogen during transformation under different conditions.

but must be due to an intermediate inactive substance which later is converted into active pepsin.

#### EXPERIMENTAL PROCEDURE

The solutions in all four parts of Fig. 5 contained 0.1 mg protein nitrogen per ml and the changes in pepsinogen and pepsin were determined by the procedures outlined in the Experimental methods.

The solutions were made up as follows

A 1 ml heat denatured and reversed pepsinogen containing 1 mg protein nitrogen per ml was added to a solution made up of 1 ml 2 N hydrochloric acid + 4 ml N/1 sodium acetate + 4 ml water. The reaction was carried out at 35.5°C

B 0.1 ml of 10 mg protein nitrogen per ml pepsinogen was added to 10 ml of M/10 pH 4.0 acetate at 25°C

C 0.1 ml of 10 mg protein nitrogen per ml pepsinogen was added to a solution made up of 5 ml 2 M magnesium sulfate + 1 ml M/1 pH 4.45 acetate and 4 ml water, all at 25°C

D 0.1 ml of 10 mg protein nitrogen per ml pepsinogen was added to 10 ml of N/1 hydrochloric acid at 0°C

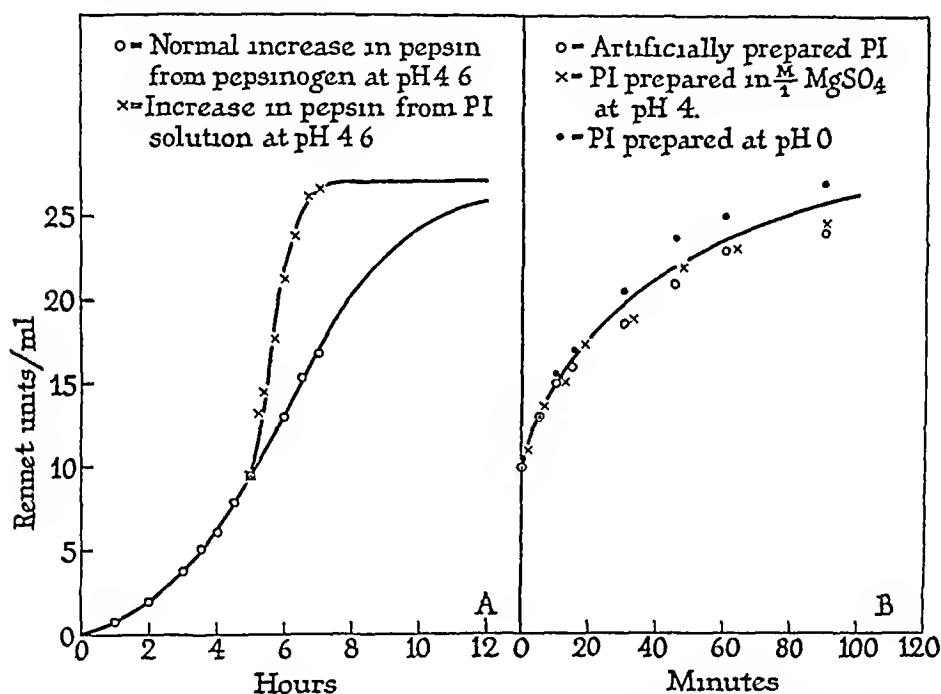


FIG 6 A Comparison of rate of formation of pepsin from pepsinogen and the intermediate compound, *PI*, at pH 4.6 B Comparison of rate of formation of pepsin from *PI* prepared by different procedures

Further evidence of an intermediate compound is shown in Fig 6 A. A pH 0 transformation mixture in which all the pepsinogen had disappeared but which contained only a small fraction of its full pepsin activity was shifted to pH 4.6 and the rate of pepsin formation examined. This was compared with the rate of formation of pepsin in a similar pepsinogen solution which had been at pH 4.6 throughout the entire reaction. It may be seen in Fig 6 A that pepsin is formed

in the two solutions at entirely different rates and therefore the two solutions must contain different substances. At pH 0, therefore, an intermediate compound, *PI*, accumulates in the solution and at pH 4.6 this dissociates into pepsin and inhibitor.

In Fig. 6B are the curves showing the rate of pepsin formation from the intermediate compound called *PI* prepared in three quite different ways. Since the materials change into pepsin at about the same rate it is probable that they are the same substance.

#### EXPERIMENTAL PROCEDURE

*A Normal Transformation at pH 4.6*—1 ml. of dialyzed purified heat denatured and reversed pepsinogen solutions containing 1 mg. protein nitrogen per ml. was added to a solution made up of 1 ml. 2 N hydrochloric acid, 4 ml. N/1 sodium acetate, and 4 ml. water. The solution was kept at 35.5°C and analyzed at varying intervals of time for active pepsin by the rennet method after a dilution of 0.1/10 in water.

*Transformation of (PI) at pH 4.6*—1 ml. of the same pepsinogen as used in the above experiment but cooled to 0°C was added to 1 ml. 2 N hydrochloric acid also at 0°C. This was kept at this temperature for 10 minutes after which 8 ml. of cold N/2 sodium acetate was mixed with it. The tube containing the solution was placed at 35.5°C and analyzed immediately for pepsinogen and at varying intervals of time for pepsin activity as in the above experiment. The pepsinogen content was only 10 per cent of the original amount, i.e., there had been a 90 per cent conversion.

#### *B Artificially Prepared PI*

*Inhibitor*—145 ml. 1 mg. protein nitrogen per ml. pepsinogen at room temperature pH 6.5 + 3 ml. 0.2 N hydrochloric acid for 30 minutes at room temperature, 1.5 ml. N/1 sodium hydroxide added and solution left 10 minutes after which 40 ml. N/1 hydrochloric acid added and solution allowed to stand 30 minutes, suspension filtered. Filtrate titrated to pH 3.5. Analysis showed the presence of no pepsin activity in this filtrate.

*PI*—0.5 ml. of dialyzed purified pepsin from pepsinogen solution was added to 1 ml. N/1 sodium acetate and 2 ml. of the above described inhibitor solution pH 6.0 (yellow to methyl red and brom thymol blue). After 90 minutes at 25°C this solution was cooled to 0°C and mixed with a solution of the following composition at 35°C: 1 ml. 4 N hydrochloric acid, 11.5 ml. water, 2 ml. 2 M magnesium sulfate, and 1.9 ml. 4 M sodium acetate. This solution after thorough mixing was brought to 35.5°C and samples removed and diluted 0.1/10 in water and analyzed immediately after dilution for pepsin by the rennet method.

*PI Prepared from Pepsinogen at pH 4.0 in the Presence of M/1 Magnesium Sulfate*—1 ml. dialyzed fractionated reversed heat denatured pepsinogen containing 2 mg. protein nitrogen per ml. was mixed with 1 ml. 4 M pH 4.6 acetate and 2 ml. 2 M magnesium sulfate and allowed to stand at 0°C for 155 minutes.



This solution was then mixed with a solution at 35.5°C containing 2 ml 2 M sodium chloride, 2 ml 2 M pH 4.6 acetate, and 12 ml water. After thorough mixing 0.1 ml samples were removed, diluted to 10 ml in water, and activity determinations made as in above solution.

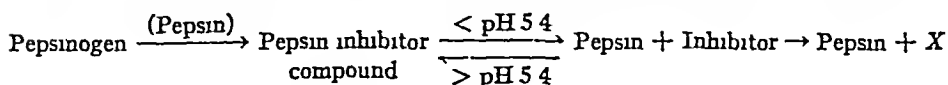
*PI Prepared from Pepsinogen at pH 0*—1 ml of the pepsinogen used in the above experiment containing 2 mg protein nitrogen per ml was added to 1 ml 4 N hydrochloric acid and 2 ml water at 0°C where it was kept for 10 minutes. A solution made up of 2 ml 2 M magnesium sulfate, 2 ml 4 M sodium acetate, and 12 ml of water at 35°C was added. There was less than 7 per cent of the original pepsinogen left, i.e., there was 90 per cent conversion. Samples were analyzed as in the above experiments after dilution of 0.1/10 in water.

### *General Nature of the Intermediate Compound, PI*

The amount of demonstrable pepsin activity in activation mixtures below pH 4 varies with the dilution, time, and pH of the solution used for measurement. This suggested the existence of a dissociable pepsin inhibitor compound as the intermediate compound. It was found that if solutions which had developed full pepsin activity were titrated to pH 6.0 the activity decreased slowly with time, suggesting that the dissociation of the pepsin inhibitor compound was reversible and a function of pH. This is actually the case and the midpoint in the dissociation equilibrium is the vicinity of pH 5.4 with complete dissociation in solutions more acid than pH 4.0 and nearly complete association at pH 6.0.

### *Mechanism of the Transformation*

The entire reaction may be represented schematically as follows



The first reaction from pepsinogen to pepsin inhibitor compound is catalyzed by pepsin while the second reaction from the compound to free pepsin and the inhibitor is a reversible dissociation. The third reaction is the destruction of the inhibitor in the presence of pepsin.

Since the pepsin inhibitor compound can be demonstrated in transformation mixtures at pH 0–pH 4, it cannot be supposed that the pepsin and inhibitor are first formed separately in solution and then combine to form *PI* compound, for the equilibrium at these pH is in

favor of the dissociated free pepsin and the enzyme, *P*, should therefore not combine with the inhibitor, *I*

At pH 4.6 where the changes in pepsin and pepsinogen are equivalent the first reaction is the slowest and determines the rate with which pepsin is produced, i.e., the *PI* dissociates liberating active pepsin as fast as it is formed. In solutions more acid than pH 4.0 the first reaction is faster than the second or dissociation reaction. Consequently the dissociation reaction determines the rate of formation of free pepsin and accounts for the accumulation of the *PI* compound in the solution.

### *General Nature of the Inhibitor, I*

The inhibitor may be prepared free of pepsin by alkali denaturation and acid precipitation of the pepsin in a freshly activated solution. When the filtrate containing the inhibitor is mixed with solutions of crystalline pepsin at pH 6.0 the pepsin activity decreases slowly. This change may be reversed by mere acidification of the solution. If, however, the activation solution is allowed to stand at pH 2.0–5.0 for a number of hours the inhibitor slowly disappears. In the absence of pepsin the inhibitor is quite stable over this range. It is stable and soluble in boiling 2.5 per cent trichloroacetic acid, insoluble in 0.7 saturated ammonium sulfate containing 2.5 per cent trichloroacetic acid, and dialyzes only slowly through collodion.

### EXPERIMENTAL METHODS

*Rennet Activity Measurements*—The rennet method was carried out as described previously (1) using "20 per cent Klum" in  $M/10$  pH 5.0 acetate buffer. The Klum solution was prepared at least 12 hours before used and discarded 2 days after preparation. In a given experiment all samples were diluted to the same volume and unless the concentration of acid or buffer was too high the solvent was distilled water. The estimation was made immediately after diluting unless otherwise stated in the procedure of the particular experiment.

*Determination of Pepsinogen*—This measurement involves the alkali denaturation of pepsin and pepsin inhibitor compound at pH 8.65 and room temperature for 15–30 minutes followed by full activation of the pepsinogen at pH 1.0–2.0 and activity estimations of the resulting pepsin. The solutions used and technique varied somewhat depending on the concentration of buffer and acid in the sample containing pepsinogen which was made alkaline. When the sample contained as much as 1 *N* hydrochloric acid as in mixtures at pH 0 so that an equivalent of alkali was required the alkaline solution was kept at 0°C until mixing and neu

trahization were complete. This diminished secondary effects due to localization of the strong alkali.

The details may best be given by the following example. 0.5 ml sample of activation mixture was mixed with 1.5 ml of  $N/3$  pH 8.65 borate buffer containing alkali equivalent to acid in the sample. This solution remained at  $25^{\circ}\text{C}$  for 15–30 minutes after which 1 ml of  $N/2$  sulfuric acid was added or sufficient acid to make the solution just pink to thymol blue. After standing 30 minutes at  $25^{\circ}\text{C}$  this solution was diluted in water and analyzed by the rennet method or hemoglobin method. The analyses by both methods are quantitatively comparable. Since the estimation of pepsin in the original activation mixture was and must be carried out by the rennet method all of the measurements in the determination of pepsinogen in the present work were made by the rennet method.

*Determination of Pepsin in the Transformation Mixture*—Estimation of pepsin in a transformation mixture was made by merely diluting a sample in water or dilute acetate buffer of such concentration and pH that the pH of the dilution did not get above pH 4.65. The estimations were made immediately after diluting and mixing.

*Pepsinogen Preparations*—Nearly all pepsinogen used in this work was prepared as previously described (1) but without crystallization. The preparation was dialyzed against dilute pH 6.8 phosphate buffer and then frozen and dried as described previously (1). In this way salt and buffer free stable materials of reproducible nature were used.

*pH*—pH estimations unless otherwise noted were colorimetric using indicators of Clark and Lubs.

#### SUMMARY

A study of the kinetics of the transformation of swine pepsinogen into pepsin under a variety of conditions has been made. The results show that the transformation as a whole is essentially autocatalytic in nature under all conditions.

Evidence is presented to show the existence of a compound intermediate between pepsinogen and pepsin. This compound was found to be a reversibly dissociable complex of pepsin and a low molecular weight inhibitor.

Some of the general properties of the intermediate compound and of the inhibitor have been examined.

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# THE ESTIMATION OF PEPSIN, TRYPSIN, PAPAIN, AND CATHEPSIN WITH HEMOGLOBIN

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In the hemoglobin method for the estimation of proteinase, denatured hemoglobin is digested under standard conditions, the undigested hemoglobin is precipitated with trichloroacetic acid, and the amount of unprecipitated protein split products, which is a measure of the amount of proteinase present, is estimated with the phenol reagent which gives a blue color with tyrosine and tryptophane

Hemoglobin, unlike casein and gelatin, is a reproducible substrate. Different batches of hemoglobin are digested at the same rate by a given proteinase solution.

Even when peptidase is present in addition to proteinase, the formation of products not precipitable by trichloroacetic acid is due so far as is known to proteinase alone.

Hemoglobin methods have been described for pepsin (Anson and Mirsky, 1932), trypsin (Anson and Mirsky, 1933), papain (Anson, 1937*a*), and cathepsin (Anson, 1937*b*). Since the methods were first worked out several minor errors have been corrected, the preparation of the hemoglobin substrates has been simplified, and the estimation procedures have been standardized. Rather than point out the numerous changes which have been made I have considered it simpler and more useful to describe completely the procedures as they are now used in this laboratory. To avoid confusion about results already published no radical changes have been made. The pepsin, trypsin, and papain substrates are the same in composition as those originally described. The cathepsin substrate now contains 0.001 M ammonium sulfate which increases the rate of digestion. In the case of the estimation of pepsin the procedure for the estimation

of the products of digestion and the calculation of the activity units have been modified to conform with the procedures used in the estimation of the other proteinases. The original method gave activity values in terms of pepsin units which are 10-15 per cent higher than the present method.

*The Preparation of Hemoglobin*—Whipped beef blood is centrifuged 20-30 minutes. The serum and the white corpuscles which form a thin layer on top of the red corpuscles are siphoned off and the red corpuscles are then mixed with an equal volume of cold 1 per cent sodium chloride solution and after centrifugation the supernatant solution is siphoned off again and the corpuscles are either stored frozen or dialyzed immediately and then stored frozen. The corpuscles are largely freed of color producing substances not precipitable by trichloroacetic acid by dialysis in Du Pont cellophane tubing of  $\frac{3}{4}$  inches diameter. Cellophane deteriorates on standing, especially the outer layer of the coil which is most exposed to the air. It is necessary to test the tubing for leaks. One end of the tubing is wetted with water and a knot is tied in the end of the tubing. The tube is then filled with water and the open end is twisted and folded over. While the folded part is pressed closed with one hand the tubing is squeezed with the other. The squeezing, in addition to showing up leaks, stretches the tubing and thus excessive stretching and dilution during dialysis are avoided. If the tubing is satisfactory the water is poured out, a marble is put in, the tube is filled with the washed corpuscles and the end of the tubing is closed with a knot in the cellophane itself. The tubes are placed in a tall vessel. Cold tap water is run into the lower part of the vessel at a rate sufficient to cause stirring. Occasionally the tubes are inverted and the hemoglobin solution is thus stirred by the marbles. After 24 hours dialysis the hemoglobin solutions from all the cellophane tubes are mixed and the mixed solution is stored frozen in small aluminum containers or cardboard ice cream containers. It is easily possible to prepare enough dialyzed hemoglobin at one time for thousands of proteinase estimations.

To estimate the concentration of protein in the dialyzed corpuscles a 3-5 gm sample is weighed out in a porcelain evaporating dish, dried

overnight at 105°C, and the dry weight recorded. The number of grams of protein per cubic centimeter of sample is

$$\frac{\text{Weight of dried protein}}{(\text{Weight of sample} - \text{weight of dried protein}) + 0.73 \text{ weight of dried protein}}$$

When it is not convenient to store the dialyzed hemoglobin frozen it can be stored at room temperature as a dry powder. If the hemoglobin solution is frozen while the drying takes place the hemoglobin remains soluble.

Bacto hemoglobin of the Difco brand can be used for rough work when the blank is not of importance. It consists of dried washed corpuscles. The other commercial hemoglobins, so far as I know, are prepared from unwashed corpuscles or from whole blood. Some of them give results very different from those obtained with the hemoglobin whose preparation has just been described.

Casein and edestin or egg albumin can be used instead of hemoglobin. Gelatin cannot be used since it is not precipitated by trichloroacetic acid.

*Phenol Reagent*—To the phenol reagent prepared according to Folin and Ciocalteu (1927) twice its volume of water is added. Whenever the phenol reagent is referred to this diluted reagent is meant.

### *The Estimation of Pepsin*

*Preparation of Hemoglobin Substrate*—The dialyzed hemoglobin solution is diluted with water to give a solution containing 2.5 gm protein per 100 cc. and is centrifuged. The small precipitate is rejected. 10 mg merthiolate (Lilly) per 40 cc is added as a preservative. Toluol should not be used nor should the amount of merthiolate be increased since larger amounts give a significant color with the phenol reagent. The 2.5 per cent hemoglobin solution is stored at 5°C.

4 cc of 2.5 per cent hemoglobin solution is added with an automatic pipette to a 175 × 20 mm test tube. Then 1 cc of 0.3 N hydrochloric acid is added from an automatic pipette. The final pH is 1.6. The acid substrate solution is stored at 5°C and used within a day or two.

since in some cases the blank increases with time. If the substrate solution is kept for more than an hour before being used, the test tube should be stoppered.

*Digestion and Color Development*—Digestion is carried out at 25°C. A convenient holder for the tubes is a piece of wood or bakelite with holes slightly larger than the diameter of the tubes. The tubes float upright in the bath. Enzyme and substrate are brought to the digestion temperature before digestion is begun.

1 ml of enzyme solution is added to 5 ml of substrate solution and the two solutions are mixed by whirling the tube. After 10 minutes 10 ml of 0.3 N trichloroacetic acid (estimated by titration) is added, the tube is shaken vigorously, and the suspension is filtered.

A filter paper such as Whatman No. 3 which does not absorb split products must be used. The color value of the split products should be the same whether the protein precipitated by trichloroacetic acid is removed by filtration or centrifugation.

To 5 ml of the digestion filtrate in a 50 cc Erlenmeyer flask are added 10 ml of 0.5 N sodium hydroxide and 3 ml of the phenol reagent. The solution is whirled during the addition of phenol reagent. Since the color formed depends somewhat on the rate at which the phenol reagent is added this rate is standardized by adding the reagent as rapidly as is possible and still have the reagent come out of the burette as drops. The color is read against the standard after 2–10 minutes.

*The Standard*—The standard tyrosine solution contains 0.0008 milliequivalents of tyrosine (0.0112 mg tyrosine nitrogen) in 5 ml 0.2 N hydrochloric acid, with 0.5 per cent formaldehyde as a preservative. The concentration of tyrosine is determined by the Kjeldahl method. 5 minutes are allowed for the color development after the addition of 10 cc of 0.5 N sodium hydroxide and 3 cc of the phenol reagent in the manner already described.

In practice a blue glass is used as a standard instead of the blue solution obtained from 0.0008 milliequivalents tyrosine. To avoid the necessity of finding a blue glass which exactly matches the color of the tyrosine-phenol reagent solution a No. 241 Corning glass filter is placed in or above the eye piece of the colorimeter. In the fairly monochromatic red light transmitted by this filter different blues

look alike. The blue glass standard is calibrated with the tyrosine standard and this calibration is frequently checked.

*Blank*—10 cc. of 0.3 N trichloroacetic acid is mixed with 5 cc. of hemoglobin solution, 1 cc. of enzyme solution is added, the tube is again shaken and the suspension is filtered. When a purified enzyme solution is used for digestion the blank is not increased by the enzyme solution and 10 cc. of 0.3 N trichloroacetic acid is added to a mixture of 5 cc. hemoglobin solution and 1 cc. water. 1 cc. of a tyrosine solution containing 0.0008 milliequivalents of tyrosine dissolved in 0.1 N hydrochloric acid containing 0.5 per cent formaldehyde is added to 5 cc. of the blank trichloroacetic acid filtrate. The color is then developed by the addition of 10 cc. sodium hydroxide and 3 cc. phenol reagent and read after 5 minutes against the standard.

*Calculations*—Color value of 5 cc. digestion filtrate in milliequivalents tyrosine =

$$\frac{\text{Colorimeter reading for standard}}{\text{Colorimeter reading for digestion filtrate}} \times 0.0008$$

Color value of 5 cc. blank filtrate =

$$\left( \frac{\text{Colorimeter reading for standard}}{\text{Colorimeter reading for blank filtrate + added tyrosine}} \times 0.0008 \times \frac{19}{18} \right) - 0.0008$$

Unless split products are added with the enzyme the blank is usually about 0.00008 milliequivalents of tyrosine.

Color value of digestion products in 5 cc. of digestion filtrate = color value of 5 cc. of digestion filtrate — color value of 5 cc. blank filtrate.

The number of activity units corresponding to the color value of the digestion products in 5 cc. of digestion filtrate is read off from the curve (Fig. 1). When the blank is constant one can omit the calculation of color values and use a curve in which the colorimeter reading for 5 cc. digestion filtrate is plotted against activity units.

### *The Estimation of Trypsin*

*Hemoglobin Substrate*—A solution is made up containing 8 cc. of 1 N sodium hydroxide, 72 cc. water, 36 gm. urea, and 10 cc. of 22 per cent hemoglobin (22 gm. hemoglobin per 100 cc. solution). This alkaline solution is kept at 25°C. for 30–60 minutes to denature the



hemoglobin and is then mixed with a solution containing 10 cc 1 M potassium dihydrogen phosphate and 4 gm of urea. The final pH is 7.5. 1 mg merthiolate (Lilly) is added to each 50 cc of hemoglobin solution as a preservative. The hemoglobin solution is stored at 5°C and is stable for weeks.

Smaller or larger quantities of substrate solution can, of course, be made up so long as the components are added in the manner and the proportions given.

The activity curve is given in Fig. 2.

The procedure for the estimation of trypsin is the same as that for the estimation of pepsin except that because of the urea in the substrate solution it is necessary after the addition of trichloroacetic acid to wait 30 minutes before filtration both in the preparation of the digestion filtrate and in the preparation of the blank filtrate.

#### *The Estimation of Papain*

The hemoglobin substrate and the estimation procedure are the same as in the estimation of trypsin. The papain must be activated before estimation.<sup>1</sup> This is done as follows. To 0.5 ml papain solution is added 5 drops 2 N sodium cyanide. After 3 minutes at 25°C 9.25 ml of water is added. If further dilution is necessary it is carried out with a solution containing 5 drops of 2 N sodium cyanide in 10 ml of water.

To obtain the papain activity curve (Fig. 3) digestion was carried out with activated aqueous extract of Optimo Papain (S. B. Penick and Company). The amounts used for digestion corresponded to 0.06–0.3 mg of the original powder. It is not known whether or not all samples of commercial papain give the same activity curve.

#### *The Estimation of Cathepsin*

*Preparation of Hemoglobin Substrate*—There is added from an automatic pipette to a 175 × 20 mm test tube 4 ml of centrifuged 2.5 per cent hemoglobin solution containing 1 mg merthiolate per

<sup>1</sup> The activation procedure described gives more rapid and complete activation of crude commercial papain than the usual activation in less alkaline solution. It has not been proved that it gives complete activation. The cyanide is necessary to eliminate inhibitors in the hemoglobin solution as well as to activate the enzyme. The amount of cyanide needed for this purpose may vary with different hemoglobin preparations.

40 cc (the same solution used for the preparation of the pepsin substrate) From another automatic pipette 1 ml of a solution 1.35 M in respect to acetic acid and 0.02 M in respect to ammonium sulfate is added. The final pH is 3.5. The acid substrate solution is stored at 5°C and used within a day or two before the blank increases.

The estimation procedure is the same as that used in the estimation of pepsin except that the digestion is carried out at 37°C instead of 25°C. The concentration of ammonium sulfate in the enzyme solution should not be greater than 0.04 M (0.01 saturated) since ammonium sulfate in greater concentration decreases the rate of digestion.

Purified beef spleen cathepsin was used to obtain the activity curve (Fig. 4).

#### *Activity Units and the Construction of the Curves*

This section defines the activity units and gives the directions for constructing a curve relating activity units to color values of digestion products. It is not necessary to read this section in order to use the procedures for the estimation of pepsin, trypsin, papain, and cathepsin which have been described, since the curves are already given. If the hemoglobin method is applied to other proteinases, however, a new curve must be worked out in each case and, in each case, the proper substrate solution must first be found.

One unit of proteinase is defined as the amount which digests hemoglobin under the standard conditions at an *initial* rate such that there is liberated per minute an amount of split products not precipitated by trichloroacetic acid which gives the same color with the phenol reagent as 1 milliequivalent of tyrosine. This unit is similar to the other proteinase units previously used in this laboratory (Northrop 1932). The specific activity is the activity per milligram of enzyme nitrogen. The standard temperatures are taken to be 25°C for papain and 37°C for cathepsin, these being the temperatures used in practice. In the cases of pepsin and trypsin, the first two proteinases studied, although in practice the digestion is carried out at 25°C the standard temperature was taken to be 35.5°C that being the standard temperature previously used in this laboratory. The initial rate of digestion by pepsin is 1.82 times greater at 35.5°C than at 25°C. With trypsin the rate is 1.78 times greater at 35.5°C than at 25°C.

To obtain an activity curve, the hemoglobin solution is digested for 10 minutes with different amounts of enzyme expressed as cubic centimeters of some stock solution. A curve is plotted relating the color values of the digestion products, *i.e.* the color values of the digestion filtrates corrected for the blanks, to the amounts of enzyme used. A line is drawn tangent to the first part of the curve and there is read off from this line the amount of enzyme which gives a color value of 0.001 milliequivalents of tyrosine for 5 cc. or  $\frac{5}{16}$  of the whole trichloroacetic acid filtrate after 10 minutes of digestion. This amount of enzyme multiplied by  $1000 \times \frac{5}{16} \times 10$  is the one unit of enzyme which produces split products with a color value of 1 milliequivalent of tyrosine in the whole 16 cc. of solution per minute.

The number of activity units per cubic centimeter of stock solution is now known and one can replot the curve to give activity units against color values.

A convenient practical hemoglobin unit which has no theoretical significance or theoretical relation to units previously used is this. When hemoglobin is digested for 10 minutes by 1 practical hemoglobin unit of proteinase at the temperature regularly used the color value of the digestion products in 5 cc. of trichloroacetic acid filtrate is 0.0008 milliequivalents of tyrosine. For each proteinase an empirical curve must be obtained relating activity units to color values. It is not necessary to obtain the first part of the curve which is not used in practice and which cannot be obtained as accurately as the middle portion of the curve. The curves given for the standard units can be used to calculate the curves for the practical units.

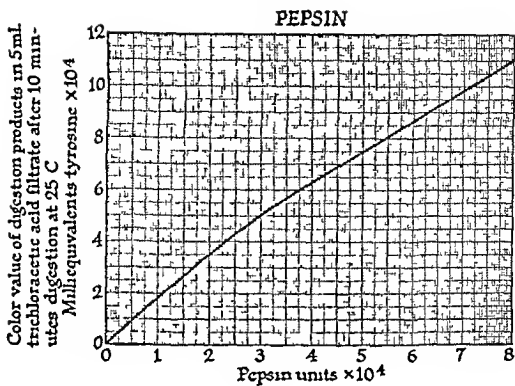


FIG 1

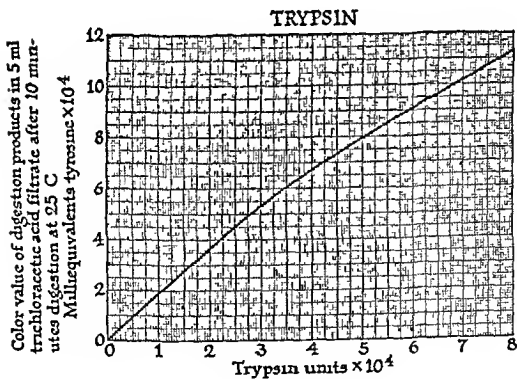


FIG 2

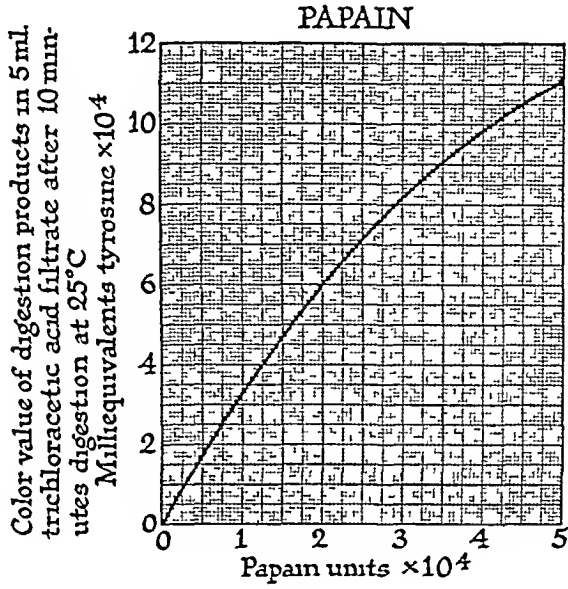


FIG 3

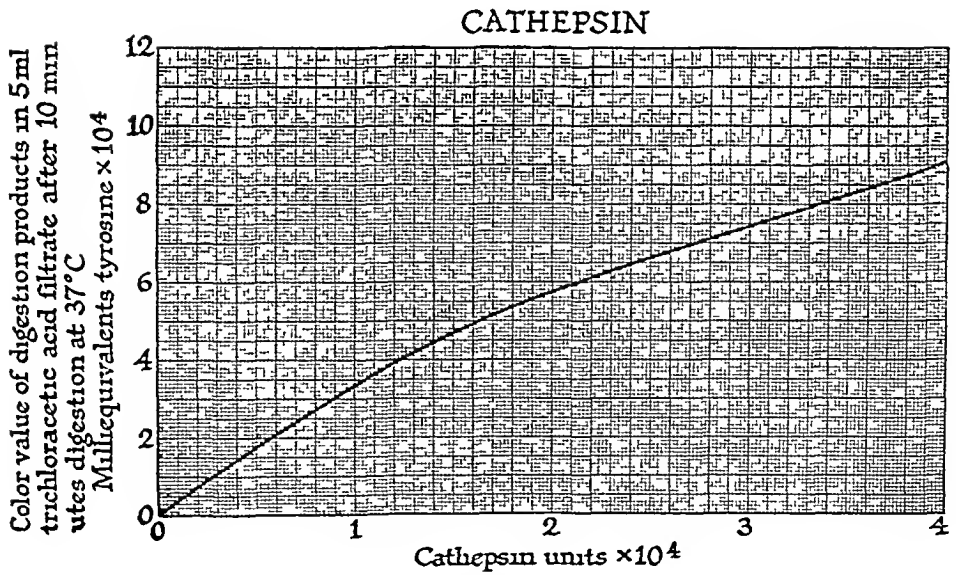


FIG 4

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✓Folin, O , and Ciocalteau V 1927 *J Biol Chem* , 73, 627  
Northrop J H , 1932, *J Gen Physiol* , 16, 41



# NATURE OF THE ACTION CURRENT IN NITELLA

## IV PRODUCTION OF QUICK ACTION CURRENTS BY EXPOSURE TO NaCl

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To understand the nature of the action current we must bring it as far as possible under experimental control

Several methods have already given promising results<sup>1</sup> with *Nitella*. One of these consists in treating cells with NaCl

The usual action curve of *Nitella* is shown in Fig 1. It has been suggested<sup>2</sup> that it depends on the movement of  $K^+$  and if this can be manipulated it should be possible to control the action current. An attempt in this direction has met with some success. The principle involved is as follows:

The normal action current appears to flow outward from the sap across the protoplasm, then lengthwise, chiefly in the cellulose wall, and back into the sap (Fig 3, at *a*). In crossing the protoplasm it passes through an aqueous layer II bounded by very thin non-aqueous layers I and I', whose resistance is undoubtedly very high. If II' could be made more conductive so that the lengthwise flow would take place in it (Fig 3 at *b*) rather than in the cellulose wall we might expect more rapid recovery and action curves with a single peak.

<sup>1</sup> Unpublished results. See also Osterhout W J V and Hill S E. Some ways to control bioelectrical behavior in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor Long Island Biological Association 1936 4, 43. Hill, S E. *Biol Bull* 1937-38 73, 362.

The experiments were performed on *Nitella flexilis* Ag. using the technique described in a former paper (Hill S E and Osterhout W J V. *J Gen Physiol* 1937-38 21, 541). Temperature 18-25 C.

The cells after neighboring cells had been cut away were placed in Solution A for some days. They were transferred to 0.01 M NaCl for half an hour or more and then placed on paraffin blocks using flowing contacts or cup technique as previously described.

<sup>2</sup> Osterhout W J V. *J Gen Physiol* 1934-35 18, 215.



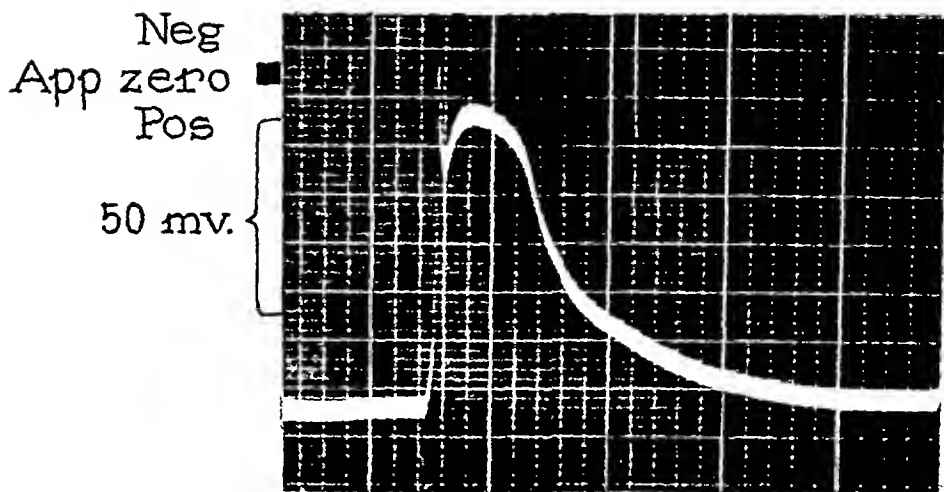


FIG 1 Shows a normal monophasic action current The spike goes to zero as frequently happens (In this case the zero is only approximately known since it is assumed that in contact with  $0.01 \text{ M}$  KCl the P.D. is zero (as is usually the case) hence it is labelled "App zero") This applies to all the other figures except Fig 8

The leads were arranged as shown in Fig 2 The record of *D* in contact with  $0.01 \text{ M}$  NaCl is shown that of *C* and *E* (omitted to save space) shows that no change occurred at *F*, which was in contact with  $0.01 \text{ M}$  KCl

Heavy time marks 5 seconds apart Temperature  $23^{\circ}\text{C}$

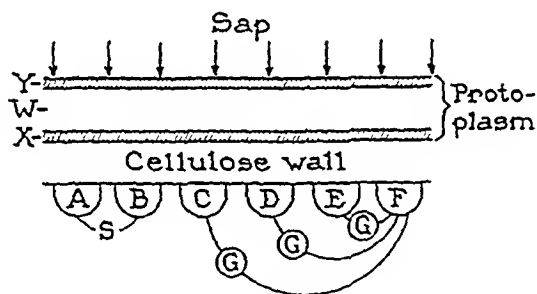


FIG 2 Diagram to show the arrangement of leads and the hypothetical structure of the protoplasm

The aqueous layer *W* is bounded by the non-aqueous layers *X* and *Y* The arrows show the P.D. at *Y* supposedly due chiefly to an outward gradient of  $\text{K}^+$  across *Y* In addition there may be in some cases an outward gradient at *X*

The electrical stimulus consists of a current entering at *A* and going out at *B*

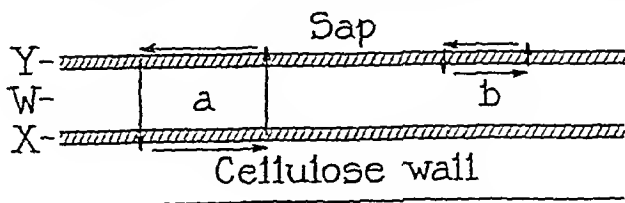


FIG 3 Hypothetical diagram to show the course of the normal action current (*a*) and the course of the quick action current (*b*)

Both of these expectations are realized when we expose the cell for half an hour or more to 0.01 M NaCl which presumably makes II more conductive.

Let us now consider some of the important factors in this situation.

The sap contains about 0.05 M KCl and 0.05 M NaCl. The effect<sup>4</sup> of  $K^+$  is so great that other ions may be neglected in the present discussion.

The outward flow of the action current means an outward movement of  $K^+$  and other cations. This may have the following consequences.

(a) *Double and Single Peaks*—The double peak of the typical action current may be due to the following.

*The o Movement*—When  $K^+$ , moving outward, reaches the outside of I the concentration gradient of  $K^+$  across V (on which the P.D. depends) is abolished and in consequence there is a loss of P.D. (negative movement or spike of the action curve) (Fig. 4).

*The p Movement*—When  $K^+$  reaches the inside of A it will create an outwardly directed P.D. (positive movement of the action curve). With the completion of o and p we have the first peak of the curve.

*The q Movement*—When  $K^+$  reaches the outside of V the positive P.D. across V disappears more or less completely (second negative movement of the curve).

*The r Movement*—The process of recovery now sets in, moving  $K^+$  inward across V, i.e. back toward the sap and thus reversing the q movement. Thus a positive movement occurs and this together with the q movement makes the second peak.

On theoretical grounds it might be predicted that soaking the cell in a solution of NaCl would make the protoplasm more conductive so that  $K^+$  would flow mostly in II' and not much of it would reach V, hence the q movement would not occur and the curve would have only one peak.

(b) *Rapidity*—This depends on the time of recovery which might be regarded as the time required to move  $K^+$  back into the sap. But if  $K^+$  merely moved into II' instead of into the cellulose wall it could return more quickly and thus shorten the recovery time.

<sup>4</sup> This appears to be due to a combination of a high mobility and a high partition coefficient. Even when the mobility of  $Na^+$  becomes relatively high it has much less effect than  $K^+$  because the partition coefficient of the latter is so high.

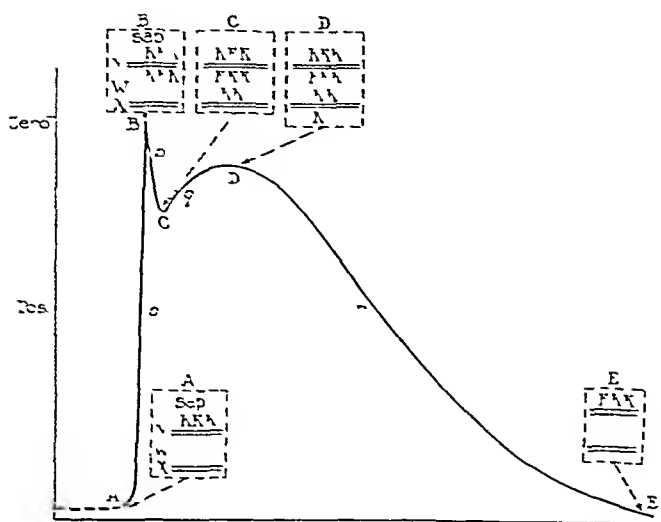


FIG 4 The unbroken line shows change in P.D. during the action current in *Nitella* supposedly due to the outward movement of potassium. The broken line shows the P.D. in the resting state before the outward movement of potassium begins.

In the diagrams the symbol K denotes the outward moving potassium (reduction in its concentration is shown by reduction in the number of symbols). Each stage of its progress is marked by a change in P.D. for example, in Diagram A the observed P.D. is supposedly due to the gradient of potassium across  $I'$ , in Diagram B we see that potassium has reached the outer surface of  $I'$  and in consequence the gradient and the P.D. have disappeared.

The duration of the action current is usually about 15 to 30 seconds.

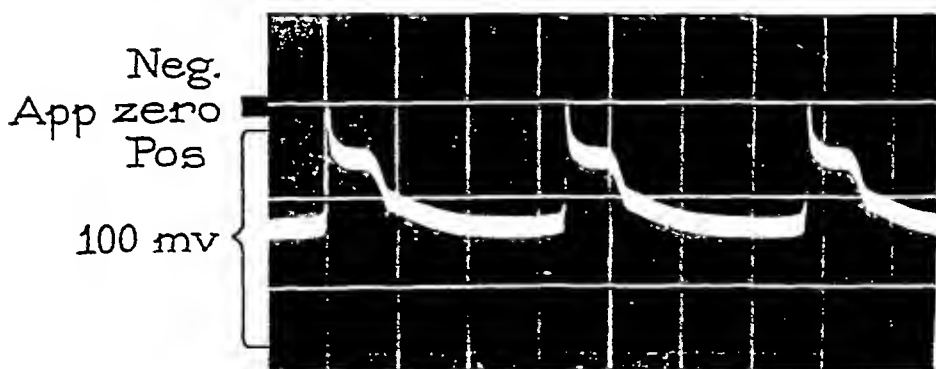


FIG 5 Shows transitional forms of action current after exposure to 0.01 M NaCl. The record of E (in contact with 0.01 M NaCl) is shown (cf. Fig. 2) those of C and D (omitted to save space) show that no change occurred at F, which was in contact with 0.01 M KCl.

Heavy time marks 5 seconds apart. Temperature 25°C.

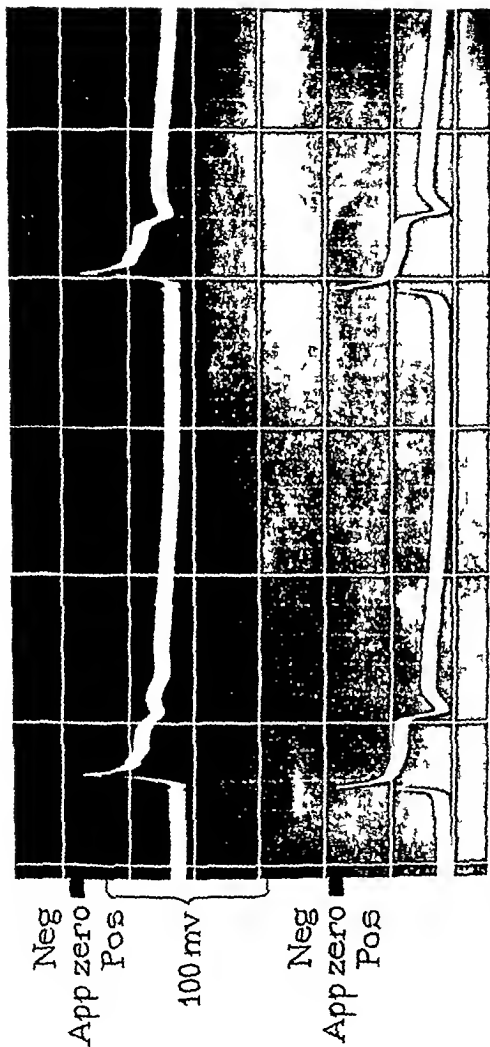


FIG 6 Shows transitional forms of action current after exposure to 0.01 M NaCl. The records of  $D$  and  $E$  (in contact with 0.01 M NaCl) are shown (cf Fig 2) there are no simultaneous movements indicating changes at  $I$  which was in contact with 0.01 M KCl ( $I$  acts as pacemaker)

The response at  $D$  (upper string) differs somewhat from that at  $L$  (lower string)  
Heavy time marks 5 seconds apart Temperature 25°C

It is possible that the outgoing  $K^+$  may move only a very short distance into  $W$  before reversing its direction and returning to the sap. The whole process may be very rapid because  $V$  presumably becomes very permeable when the action current occurs.<sup>3</sup> The increase in the permeability of  $V$  appears to be favored by NaCl since its application lowers the voltage<sup>5</sup> needed for stimulation, which may indicate that  $V$  more easily becomes permeable. We also find that the absolute refractory period is shortened by exposure to NaCl. These changes favor the production of rapid action currents.

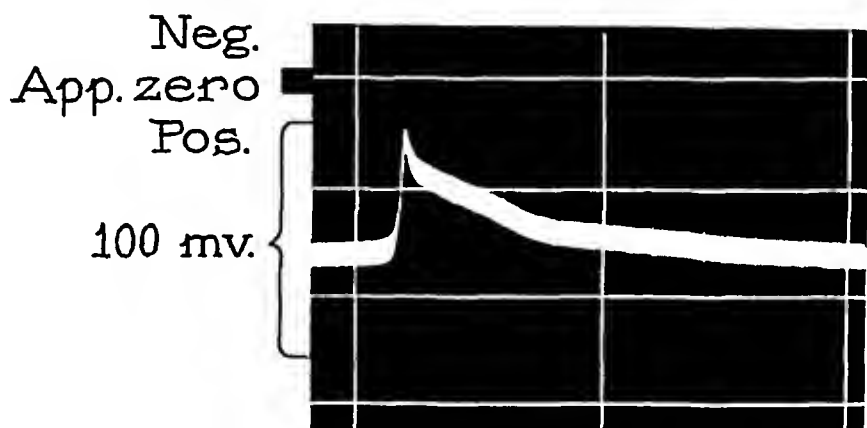


FIG 7 Shows a transitional form of action current after exposure to 0.01 M NaCl. The record of  $D$  is shown (cf Fig 2) those of  $C$  and  $E$  (omitted to save space) show that no change occurred at  $F$ , which was in contact with 0.01 M KCl. Heavy time marks 5 seconds apart. Temperature 25°C.

Various transitional forms between the normal and the rapid action currents are shown in Figs 5 to 8. Whether all of these are produced by each cell in a fixed order is not known.

The beginning of quick movements is seen in Figs 9 and 10. These usually occur spontaneously after a time but somewhat prior to this they can as a rule be induced by a brief electrical stimulation (300 mv DC applied for about 0.1 second). It will be noted that the quick movements appear to be superimposed on a slow action curve conforming more or less to the normal pattern.

In Fig 11 we see the beginning of a long train of action currents.

<sup>5</sup> Although the voltage is lowered the resistance of the cell decreases so as to compensate and permit a flow of current which is not very different.

Neg  
App zero  
Pos

100 mv

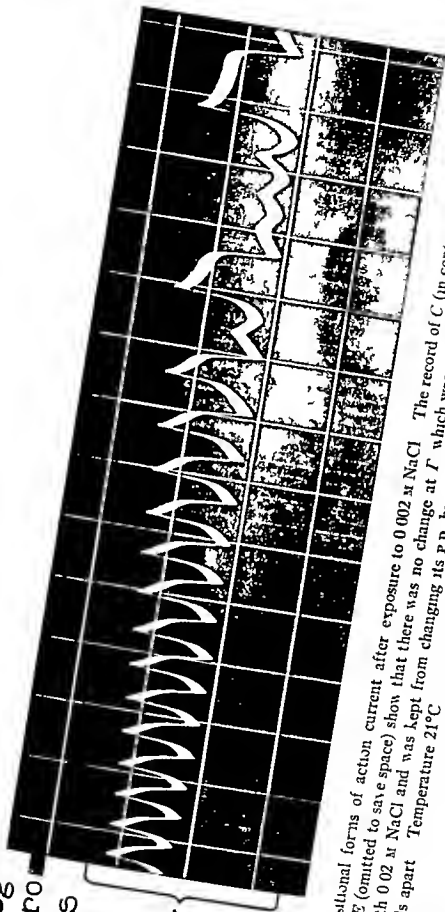


FIG 8 Shows transitional forms of action current after exposure to 0.002 M NaCl. The record of C (in contact with 0.02 M NaCl) is shown those of D and E (omitted to save space) show that there was no change at F which was in contact with 0.02 M NaCl (cf Fig 2). Time marks 5 seconds apart. Temperature 21°C.

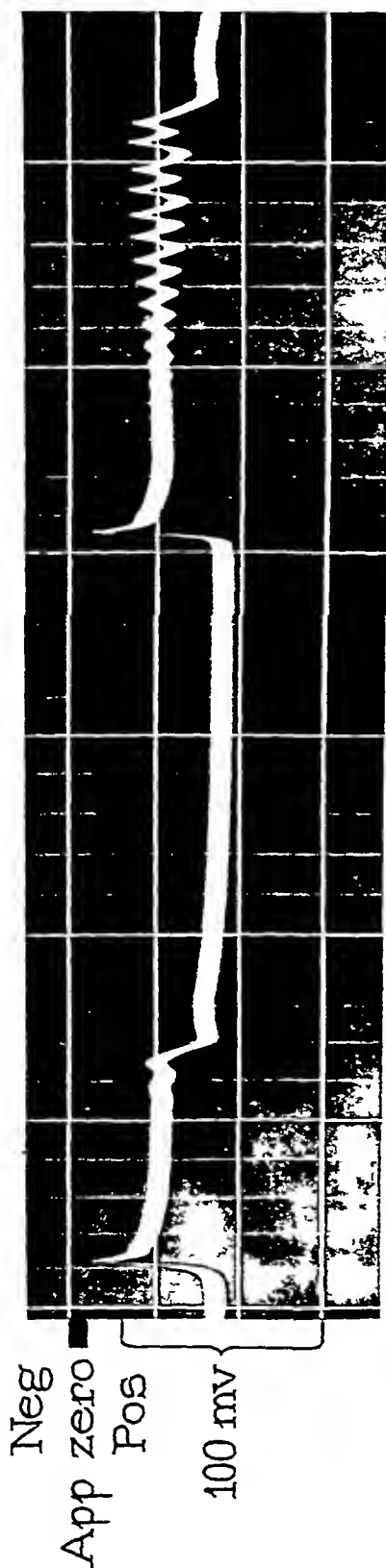


FIG. 9 Shows the beginnings of quick action currents after exposure to 0.01 M NaCl. The record of  $D$  (in contact with 0.01 M NaCl) is shown (cf. Fig. 2) those of  $D$  and  $I_2$  (omitted to save space) show that no change occurred at  $I_2$  (in contact with 0.01 M KCl). Heavy time marks 5 seconds apart. Temperature 25°C.

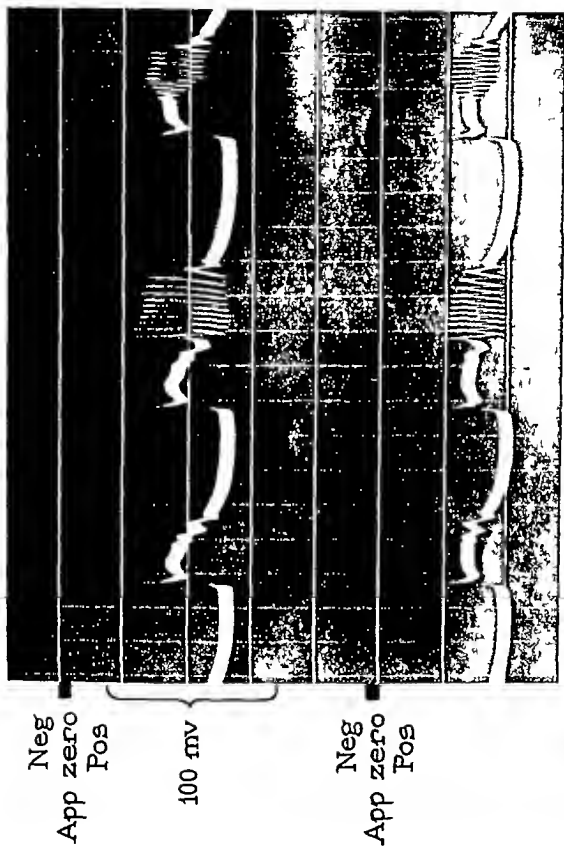


FIG 10 Shows the beginnings of quick action currents after exposure to 0.01 M NaCl. The records of C and D (in contact with 0.01 M NaCl) are shown (cf Fig 2) that of L (omitted to save space) shows that no change occurred at  $t'$  which was in contact with 0.01 M KCl. Heavy time marks 5 seconds apart. Temperature 25 C.



Such trains may go on without interruption until several hundred have appeared. But as a rule they are interrupted by intervals of rest<sup>6</sup>. Portions of such trains are seen in Figs 12 and 13. In Fig 12 the action curves are almost identical at *C*, *D*, and *E* (Fig 2) but in Fig 13 this is not the case.

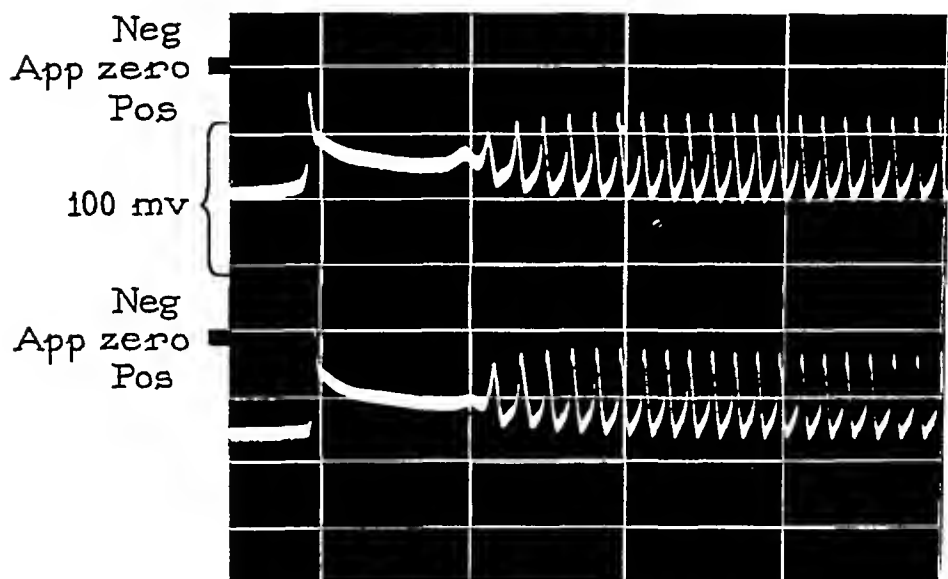


FIG 11 Shows the beginnings of quick action currents after exposure to 0.01  $M$  NaCl. The records of *C* and *D* (in contact with 0.01  $M$  NaCl) are shown (cf Fig 2) the absence of any simultaneous movements indicates that no change occurred at *F*, which was in contact with 0.01  $M$  KCl.

The pacemaker is at the left of *C*, as shown by the fact that in each action current the upper string moves first. The responses at *C* (upper string) and *D* (lower string) are very similar.

Heavy time marks 5 seconds apart. Temperature 25°C.

It happens that the upstroke is slower than the downstroke in these figures, and Fig 14 indicates that in some cases at least the upstroke involves a certain hesitation when the "base line," i.e. the resting potential, is reached. The downstroke in this figure

<sup>6</sup> An example is shown in a previous paper (Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934-35, 18, 512 (Fig 15)). In this case the exposure to NaCl was so short that we may suppose that *W* was already more conductive than usual when the cell was placed in NaCl.

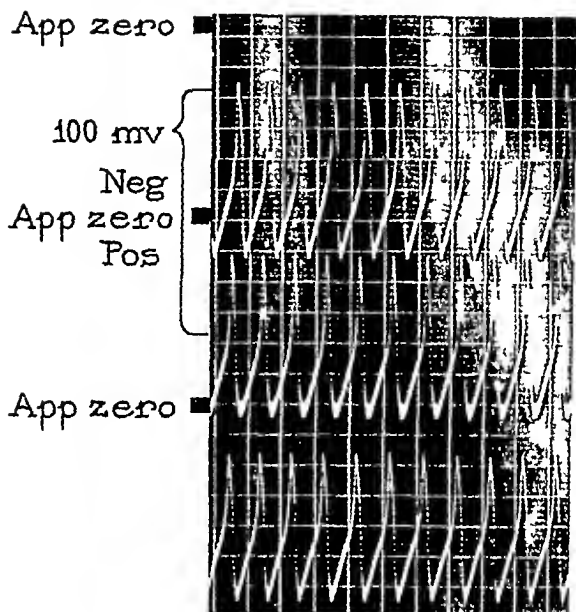


FIG 12 Portion of a train of quick action currents after exposure to 0.01 M NaCl. The records of C, D and E (all in contact with 0.01 M NaCl) are shown (cf Fig 2) the absence of simultaneous movements indicates that no changes occurred at F, which was in contact with 0.01 M KCl.

The pacemaker was at the right of E as indicated by the fact that E (lower string) changes first in each action current. The responses are similar at each spot. Time marks 1 second apart. Temperature 23°C.

appears to go below the 'base line' and then rise, linger at the base line for a brief period, and then go on (cf Fig 13).

The shapes of the action curves have been discussed in previous

<sup>1</sup> As shown in a previous paper (Osterhout W J V and Hill S E *J Gen Physiol* 1934-35, 18, 499) there may be two base lines: one of which is the complete and the other the 'incomplete resting potential'.

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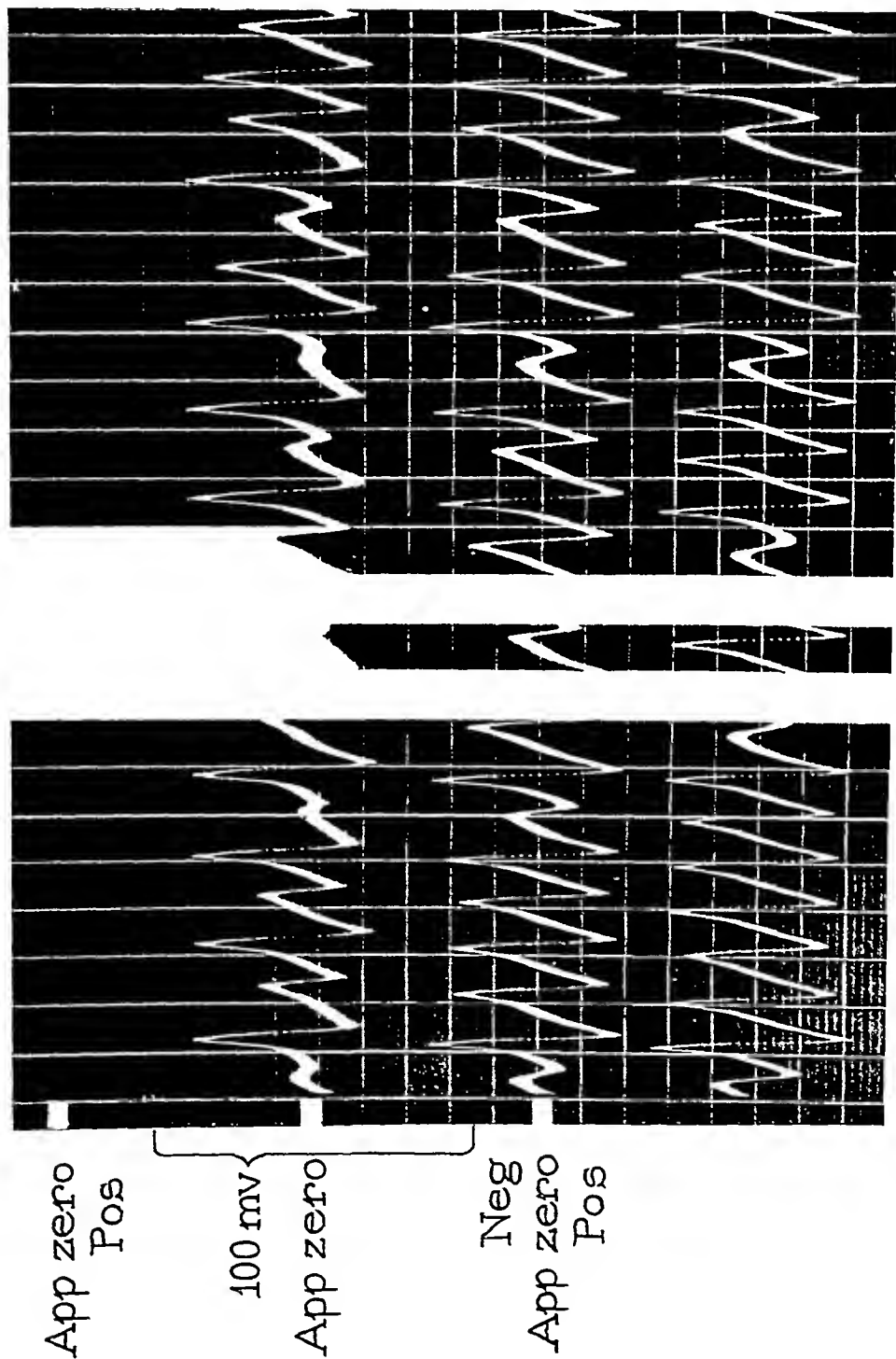


FIG 13 Portion of a train of quick action currents after exposure to 0.01 M NaCl. The records of  $C$ ,  $D$ , and  $E$  (all in contact with 0.01 M NaCl) are shown (cf Fig 2) the absence of any simultaneous movements shows that no change occurred at  $F$ , which was in contact with 0.01 M KCl.

The pacemaker was at the right of  $E$  as indicated by the fact that in each action current the first change was at  $E$  (lowest string). The responses at the three spots are not identical.

Time marks 1 second apart. Temperature 22°C.

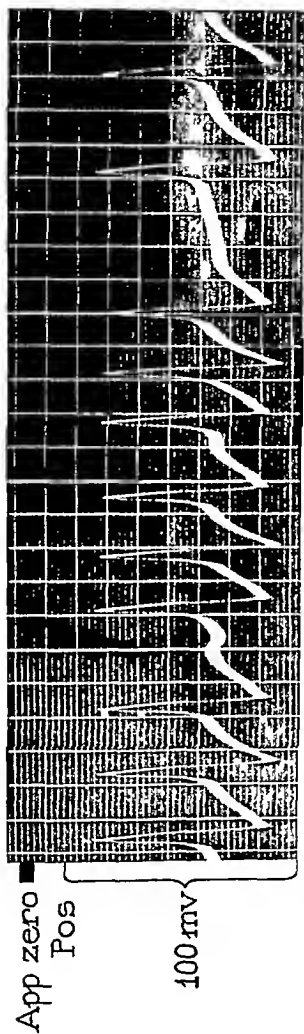


FIG. 14 Shows the tendency to lag in the upstroke as the base line or resting potential is approached (in this case there is considerable positive after potential). The record of *C* (in contact with 0.01 *M* NaCl) is shown (*cf* Fig. 2) those of *D* and *E* (omitted to save space) show that no change occurred at *E* which was in contact with 0.01 *M* KCl. Time marks 1 second apart. Temperature 22°C.

papers<sup>8</sup> which may be referred to for details. It is assumed that  $K^+$  comes out of the sap during the action current and that it goes back into the sap during recovery. If the outward movement of  $K^+$  is restricted or if it fails to reach the outside of  $X$  and to destroy any positive potential at  $X$  the curve may not go to zero. If the outgoing  $K^+$  does not return completely to the sap recovery will be incomplete. In the present experiments the curves do not go to zero and in the quick movements recovery is often incomplete (Figs 9 and 10).

It may be noted in passing that when  $F$  (Fig 2) is in contact with 0.01 M KCl it frequently appears to act as a pacemaker, as would be expected since the P.D. at  $F$  would be approximately zero<sup>9</sup> and it has been found that this condition easily sets up action currents. But changes in the location of pacemakers are often observed and when a rhythm is once established it sometimes continues even when the supposed pacemaker is changed or blocked off. The question of pacemakers will be discussed elsewhere.<sup>10</sup>

In this connection attention may be called to the long trains of action currents produced, according to Brink and Bronk,<sup>11</sup> by treating the sciatic nerve of the frog with Ringer's solution free from calcium.

The response of the cell to NaCl presents several interesting aspects among which are the following:

1 *Prolonged Exposure to NaCl*—After some hours in 0.01 M NaCl the irritability may disappear for an hour or more so that the cell can no longer be stimulated electrically.<sup>12</sup> To what extent this depends on the previous production of a large number of rapid action currents is an open question.

After some hours irritability returns and the action currents tend

<sup>8</sup> Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 377.  
Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934-35, **18**, 499.

<sup>9</sup> Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929-30, **13**, 459.

<sup>10</sup> Regarding pacemakers, see Auger, D., *Comparaison entre la rythmicité des courants d'action cellulaires chez les végétaux et chez les animaux*, *Actualités scient. et indust.*, 314, Paris, Hermann et Cie, 1936.

<sup>11</sup> Brink, F., Jr., and Bronk, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1937-38, **37**, 94.

<sup>12</sup> This refers to electrical stimulation produced in the usual way. In such cases there may be a response at the cathode which is not propagated.

to show the normal recovery time of 15 to 30 seconds. The shape of the action curves frequently resembles those in Figs 5 to 7.

Evidently a new factor enters into the situation which prevents the production of rapid action currents in spite of the presence of NaCl. This might come about if organic electrolytes were leached out of H' thus increasing its resistance.

It may be added that at this stage the cells look normal and may continue to do so even after an exposure of several weeks to 0.01 M NaCl.

2 *Effects of Calcium*—The addition of calcium suppresses the quick action currents.<sup>12</sup> If instead of 0.01 M NaCl we use a mixture of 0.01 M NaCl + 0.0005 M CaCl<sub>2</sub> no rapid action currents appear. In the presence of this concentration of calcium the voltage necessary for stimulation and the absolute refractory period do not fall off.

As might be expected no quick action currents occur in 0.005 M CaCl<sub>2</sub>.

It seems possible that the addition of CaCl<sub>2</sub> inhibits the penetration of NaCl from the external solution while NaCl moves from H' into the sap under the action of the forces which are constantly producing such a movement,<sup>14</sup> the result would be that the conductivity of H' would fall off.

It may be of interest in this connection to call attention to the experiments of Chao<sup>15</sup> who found that in *Limulus* Na<sup>+</sup> increases and Ca<sup>++</sup> decreases the rate of heartbeat when applied to the dorsal median nerve cord or ganglion where the rhythm originates.

3 *Penetration of Salts*—It is interesting to note that certain salts, such as NH<sub>4</sub>Cl and LiCl which might be expected to penetrate as readily as NaCl, have somewhat the same effect as NaCl in producing quick action currents.

We find that NaSCN which might be expected to penetrate more rapidly than NaCl produces a quicker and more pronounced effect.

But Na<sub>2</sub>SO<sub>4</sub> which might be expected to penetrate more slowly produces little or no effect. Quick action currents are produced by

<sup>12</sup> This also happens in nerve. See footnote 11.

<sup>14</sup> Cf. Osterhout W J V *Ergebn. Physiol.* 1933 35, 967 *Biol. Rev.* 1936 2, 283.

<sup>15</sup> Chao I *Biol. Bull.* 1933 64, 358.

$(\text{NH}_4)_2\text{SO}_4$  but this may be due to the penetration of undissociated  $\text{NH}_3$  ( $\text{MgSO}_4$  has no effect)

4 *Variability*—Very irritable cells (with a low threshold for electrical stimulation) quickly give rapid action currents<sup>16</sup> when placed in 0.01 M NaCl. Some cells require an exposure of only 15 minutes to 0.01 M NaCl to produce quick action currents and occasionally cells are found which require an even briefer exposure or none at all.<sup>8</sup> In these cases we may suppose that *W* is more conductive than usual owing to the presence of organic or inorganic electrolytes. In some cases we may have to do with electrolytes which have come out of the sap during an action current and have not gone back completely (incomplete recovery).

Cells with a high threshold require a longer exposure and in some cases fail altogether to produce rapid action currents.

#### SUMMARY

Treatment of *Nitella* with NaCl greatly reduces the time required for the action current and produces an action curve with one peak instead of the customary two. The time may be reduced to 0.6 second in place of the usual 15 to 30 seconds.

This might be expected if the treatment increased the conductivity of the aqueous part of the protoplasm. The experiments favor this idea although they do not prove its correctness.

This effect is prevented by calcium, possibly because calcium inhibits penetration of salts. That penetration is an important factor is indicated by the fact that salts which might be expected to penetrate rapidly have the most effect. Thus NaSCN is more effective than NaCl but  $\text{Na}_2\text{SO}_4$  has little or no effect. The action of  $\text{NH}_4\text{Cl}$  and  $\text{LiCl}$  is similar to that of NaCl.

<sup>16</sup> Cells which have been freed from neighboring cells and then kept in the laboratory for several weeks seem to be especially favorable.

## DELAYED POTASSIUM EFFECT IN NITELLA

BA S E HILL AND W J V OSTERHOUT

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication April 22, 1938)

In normal cells of *Nitella* potassium produces a large and rapid change of P.D. in a negative<sup>1</sup> direction. When 0.01 M NaCl is replaced by 0.01 M KCl the P.D. usually becomes less positive<sup>2</sup> by 80 mv or more. This is called for convenience the potassium effect (Figs 1 and 3). Since the response to KCl is so rapid we suppose that it occurs at \ the outer non aqueous protoplasmic surface layer (cf Fig 2).

The potassium effect usually disappears when cells are placed for 2 or 3 days in distilled water<sup>3</sup>. This removes organic material which may be recovered from the distilled water and returned to the cell thereby restoring the potassium effect. For convenience this substance<sup>4</sup> (or group of substances) may be called  $R_F$ .

When the potassium effect has been lost as the result of exposure to distilled water or for other reasons<sup>5</sup> the replacement of 0.01 M

<sup>1</sup> The P.D. is said to be negative when the positive current tends to flow from the external solution across the protoplasm to the sap. All the effects of KCl described in this paper were fully reversible.

Cf Osterhout W J V, *J Gen Physiol* 1929-30 13, 715

<sup>2</sup> Osterhout, W J V, and Hill, S E. *J Gen Physiol*, 1933-34 17, 105

<sup>3</sup> Exposure to distilled water also removes irritability and thus appears to be due to another organic substance (or group of substances) which may be called  $R_A$ . The reason for supposing that  $R_F$  and  $R_A$  are different is that the loss of irritability sometimes precedes and sometimes follows the loss of the potassium effect. But it is of course possible that we are dealing with a single substance operating under different conditions.

<sup>5</sup> This usually takes place under natural conditions in late spring and early summer the season of rapid growth (cf Osterhout W J V and Hill S E. *J Gen Physiol* 1933-34 17, 105) and may happen in an occasional cell at any time of year.



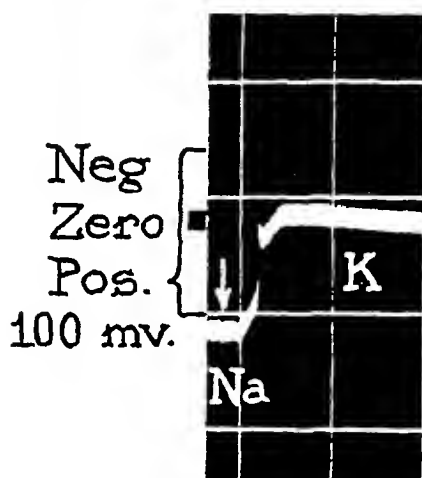


FIG 1 Shows prompt response to the application of 0.01 M KCl to a normal cell, replacing 0.01 M NaCl at the time marked by the arrow. The curve resembles an action curve. The slight delay presumably represents the time required to diffuse through the cellulose wall.

The leads were arranged as shown in Fig 2. The record shown is that of *D* (*C* and *E* were omitted). *F* was in contact with 0.01 M KCl which reduces the P.D. approximately to zero.

Heavy time marks are 5 seconds apart. Temperature 21°C.

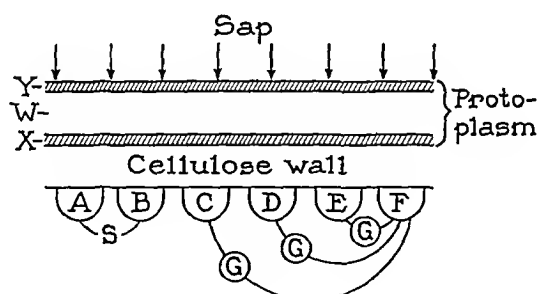


FIG 2 Diagram to show the arrangement of leads and the supposed structure of the protoplasm which is assumed to consist of an aqueous layer *W*, an outer non-aqueous layer *X*, and an inner non-aqueous layer *Y*.

The arrows show the outwardly directed (positive) P.D. whose seat is supposed to be chiefly at *Y* when the cell is in pond water; hence the P.D. at *X* is regarded as negligible and is not shown. But under some conditions the P.D. at *X* may become important.

Each lead is connected to a separate amplifier and to one string of the 3-string Einthoven galvanometer.

NaCl by 0.01 M KCl usually has little or no effect<sup>6</sup> but in some cases we obtain the result<sup>7</sup> seen in Figs 4, 5, and 6. Here the potassium effect seems to be absent when KCl is first applied but appears after some delay as though it had been restored by contact of the cell with KCl.

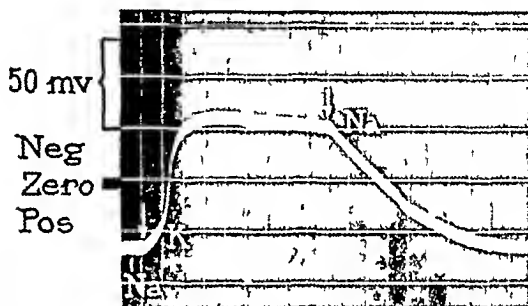


FIG. 3. Response in a normal cell showing a curve which does not closely resemble the usual action curve when 0.01 M NaCl is replaced by 0.01 M KCl. *D* (cf. Fig. 2) is recorded (*C* and *E* are omitted). Although *D* and *F* are both in contact with 0.01 M NaCl, *D* is positive to *F* owing to some local difference. *D* becomes negative to *F* as soon as KCl is applied. When 0.01 M NaCl is subsequently applied to *D* the P.D. returns to the original value.

Time marks 5 seconds apart. Temperature 28°C.

It is evident that KCl might do this if it formed a compound which sensitized *N* to the action of potassium. This compound might be  $R_p$ , formed by the reaction  $K^+ + Z = R_p$  where *Z* is an organic substance. (If  $R_p$  contains potassium it is easier to understand why it causes the cell to act somewhat like a potassium electrode.)

<sup>6</sup> If there is an effect it may be in the direction of greater or of less positivity according to the condition of the cell.

<sup>7</sup> The experiments were made on *Naella flexilis* Ag. using the technique described in a former paper (Hill, S. E., and Osterhout, W. J. V. *J. Gen. Physiol.*, 1937-38, 21, 541).

The zero in the figures is located on the assumption that 0.01 M KCl reduces the P.D. approximately to zero. This is usually close to the actual condition.

If this were the case we might expect that when KCl is applied no change in  $P D$  will occur until sufficient  $R_p$  is formed<sup>8</sup> to sensitize  $X$  and cause a gradual rise in the curve such as is seen in Figs 4, 5, and 6

In Figs 1, 4, and 6 the curve has the appearance of an action curve

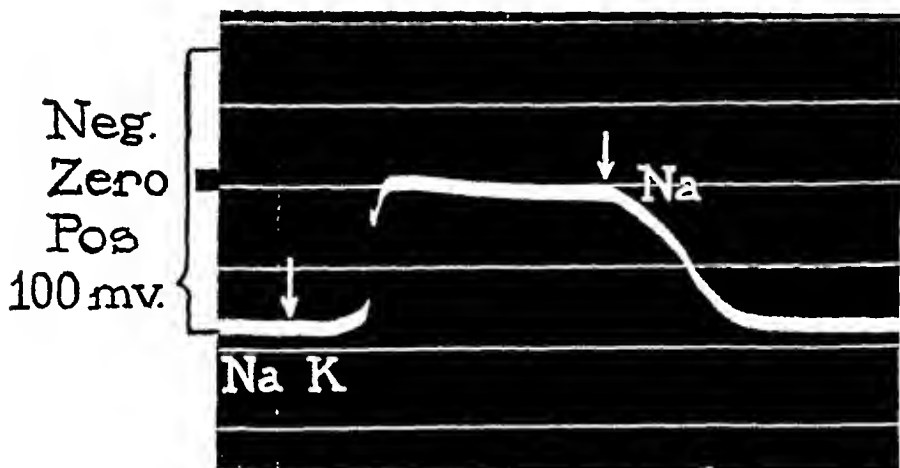


FIG 4 Shows delayed response to KCl in a cell which had been soaked for 4 days in distilled water the curve resembles an action curve At the start the spot recorded ( $D$ , Fig 2) was in contact with 0.01  $M$  NaCl When this was replaced by 0.01  $M$  KCl the  $P D$  became less positive after a delay The application of 0.01  $M$  NaCl returned the  $P D$  to the original value

The leads were arranged as in Fig 2  $C$  and  $E$  were omitted  $F$  was in contact with 0.01  $M$  KCl

Time marks 5 seconds apart Temperature 22°C

This might occur since irritability often persists after the potassium effect has disappeared

It has been suggested in a former paper<sup>9</sup> that the application of KCl at a spot  $D$  (Fig 2) might give rise to an action curve at that point by depressing the  $P D$  sufficiently to cause a discharge from a neighboring point  $D_1$  not in contact with the KCl covering  $D$  (and consequently not recorded)

The discharge at  $D_1$  would presumably involve the exit of substances

<sup>8</sup> We suppose that as a rule  $Z$  is absent since there is usually no response, not even after a delay

<sup>9</sup> Hill, S. E., and Osterhout, W. J. V. *J. Gen. Physiol.*, 1937-38, 21, 541

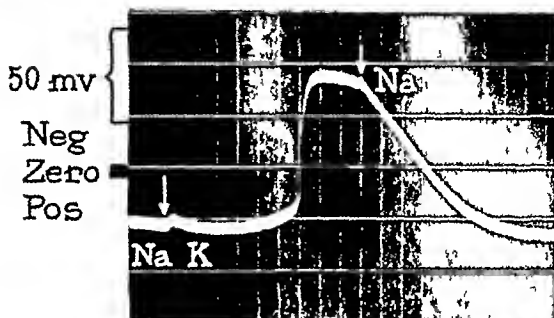


FIG. 5 Shows delayed response when 0.01 M NaCl is replaced by 0.01 M KCl the curve does not closely resemble the usual action curve. At the start the spot recorded (*D* Fig. 2) was in contact with 0.01 M NaCl. When this was replaced by 0.01 M KCl the P.D. became less positive after a delay. The application of 0.01 M NaCl returned the P.D. to the original value.

The leads were arranged as in Fig. 2. *C* and *E* were omitted. Although *F* was in contact with 0.01 M NaCl *D* was positive to it at the start (owing to some local difference) but after application of KCl to *D* it became negative to *F*.

Time marks 5 seconds apart. Temperature 28°C.

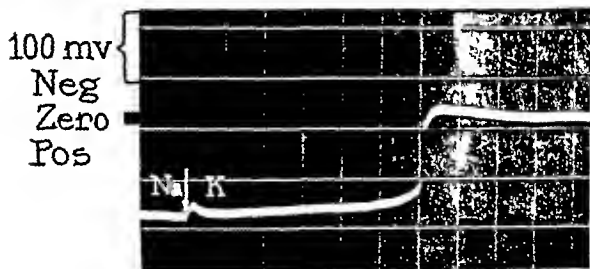


FIG. 6 Delayed response when 0.01 M NaCl was replaced by 0.01 M KCl the curve resembles an action curve and was propagated to *C* (not shown in the record). *D* is recorded (*E* was omitted). *F* was in contact with 0.01 M KCl.

Time marks 5 seconds apart. Temperature 20°C.



# PACEMAKERS IN NITELLA

## II ARRHYTHMIA AND BLOCK

By W J V OSTERHOUT AND S E HILL

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, June 4, 1938)

Irregular rhythm (arrhythmia) and failure to transmit impulses (block) seem to be essentially similar in the vertebrate heart and in *Nitella*

In *Nitella* arrhythmia and block (partial or complete<sup>1</sup>) have been produced by the following treatment. Cells (freed from neighboring cells) are kept for 6 weeks or more in a nutrient solution (Solution A). Exposure for 2 or 3 hours<sup>2</sup> to 0.01 M NaCl, 0.01 M NaSCN, or 0.01 M guanidine chloride<sup>3</sup> then reduces the time required for the action current to about 1 second (the normal time is from 15 to 30 seconds).

The leads<sup>4</sup> are shown in Fig. 1. At the common contact *F* we place 0.01 M KCl which prevents changes<sup>5</sup> at that spot, and thus renders the action currents monophasic; it reduces the P-D approximately to zero. (The location of the zero in the figures in this paper is made on the assumption that the P-D at *F* is zero, hence it is only an approximation.)

Owing to the presence of 0.01 M KCl, *F* usually acts as a pacemaker.<sup>2</sup> In the present case the rhythm is about 1 a second. Apparently certain parts of the cell are unable to follow this rapid pace and hence fall

<sup>1</sup> Complete block is sometimes found in untreated cells.

<sup>2</sup> Cf. Osterhout W J V and Hill S E, *J Gen Physiol*, 1933-34, 17, 87.

<sup>3</sup> Cf. Hill S E, and Osterhout W J V, *J Gen Physiol*, 1938-39, 22, 91.

<sup>4</sup> Unpublished.

<sup>5</sup> The experiments were performed on *Nitella flexilis* Ag. using the technique described in former papers (cf. Hill S E and Osterhout, W J V, *J Gen Physiol*, 1937-38, 21, 541).

<sup>6</sup> Any change at *F* can be detected by simultaneous changes in the records of *C*, *D* and *E*.

into irregular rhythms and fail to register all the impulses (partial block) If this does not occur and if all parts of the cell follow the rhythm perfectly, we obtain such records as are shown in Fig 2 Here the action curves<sup>7</sup> are practically the same at each of the recorded spots

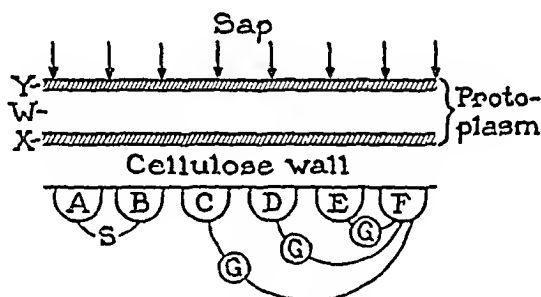


FIG 1 Diagram to show the arrangement of leads and the supposed structure of the protoplasm which is assumed to consist of an aqueous layer *W*, an outer non-aqueous layer *X*, and an inner non-aqueous layer *Y*

The arrows show the outwardly directed (positive) *P D* whose seat is supposed to be chiefly at *Y* when the cell is in pond water hence the *P D* at *X* is regarded as negligible and is not shown But under some conditions the *P D* at *X* may become important

Each lead is connected to a separate amplifier and to one string of the 3-string Einthoven galvanometer

Figs 3 and 4 show certain differences in the forms of the action curves at different spots The sudden appearance of a more striking difference is seen in Fig 5 which records action currents at *C* (top) and *D* (bottom) At first each impulse passes from *D* to *C* but later, *C* appears to be unable to follow the pace and hence it records every second impulse<sup>8</sup> This recalls a situation often found in auricular flutter of the heart<sup>9,10</sup> when the auricular rate becomes so rapid that

<sup>7</sup> Regarding the form of these curves see Hill, S E, and Osterhout, W J V, *J Gen Physiol*, 1938-39, 22, 91

<sup>8</sup> The chance of finding such cases, and of observing blocks, naturally increases as the distance between the leads increases

<sup>9</sup> White, P D, *Heart disease*, New York, The Macmillan Co, 2nd edition, 1937

<sup>10</sup> Bethe, A, *Arch ges Physiol*, 1937, 239, 41, *Z vergleich Physiol*, 1937, 24, 613 Wenckebach, K F, and Winterberg, H, *Die unregelmässige Herz-tätigkeit*, Leipsic, W Engelmann, 2nd edition, 1927

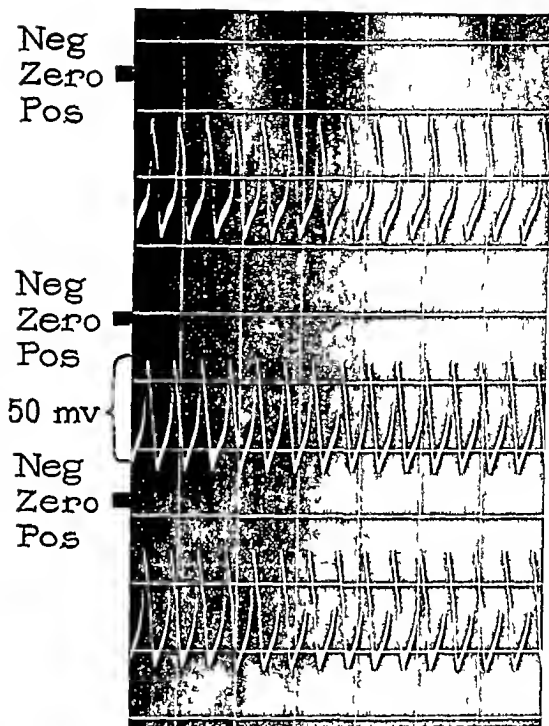


FIG 2 Shows nearly identical responses at *C*, *D* and *E* (cf Fig 1) the pace maker is at the right (probably at *F* which is in contact with 0.01 M KCl) since *E* lowest string moves first. The other spots are in contact with 0.01 M NaCl.

The cell was freed from neighboring cells and kept  $15 \pm 1^\circ \text{C}$  in Solution A. It was then placed for 3 hours in 0.01 M NaCl at  $21 \pm 2^\circ \text{C}$  before the record was made.

The leads are 6 mm apart.

Time marks 5 seconds apart.



the ventricle can record only every other impulse coming from the auricle

Such ratios as 3:1 and some others found in the heart appear in *Nitella* (cf Figs 6-8)

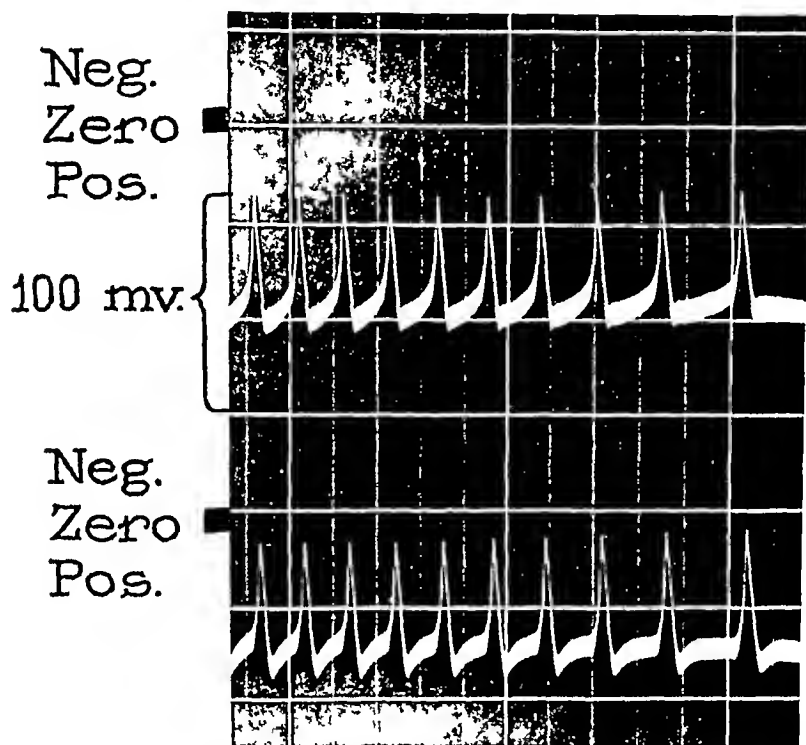


FIG 3 Shows action curves at *C* (top) and *D* (bottom, cf Fig 1) which are similar but not identical. The pacemaker is at the left of *C* (since the top string, *C*, moves first)

*F* is in contact with 0.01 M KCl, the other spots are in contact with 0.01 M NaCl. The leads are 28 mm apart.

The cell was freed from neighboring cells and kept 40 days in Solution A at  $15 \pm 1^\circ\text{C}$ , it was placed for 2.5 hours in 0.01 M NaCl at  $22^\circ\text{C}$  before the record was made.

Heavy time marks 5 seconds apart.

We also find in *Nitella* cases where one or both spots are irregular (cf Figs 7 and 8).

In Fig 9 we see that 26 action currents are recorded at *C* (top)

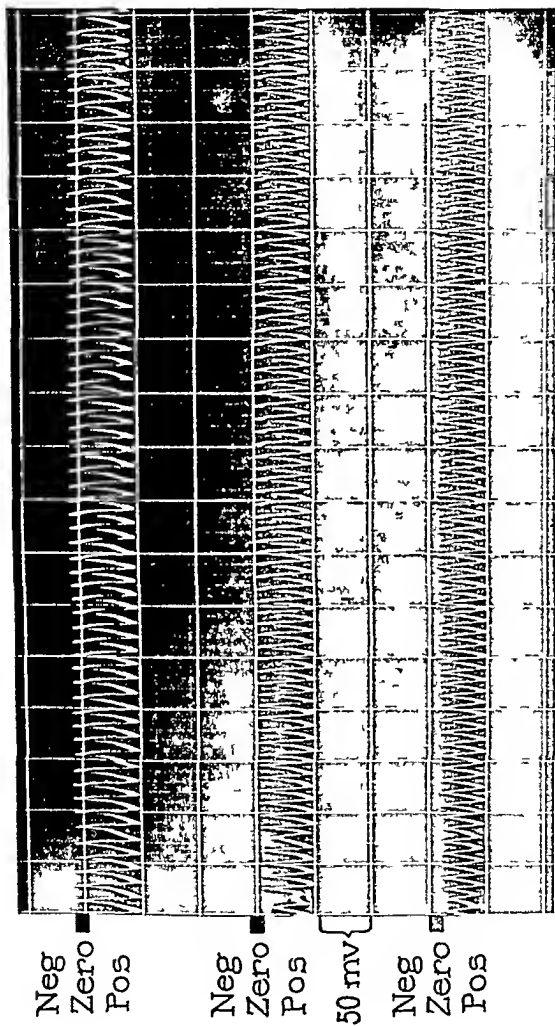


Fig 4 Shows action curves at C (top, of 1 to 1) D (middle) and L (bottom) which are similar in some cases but in others differ considerably. Since the bottom string F, moves first the piezometer is at the right of L probably at 1 which is in contact with 0.01 M KCl the other spots are in contact with Solution A. The leads are 10 mm apart.

The cell was freed from neighboring cells and placed for 50 days in Solution A at  $15 \pm 1^\circ \text{C}$ . It was then placed in 0.01 M guanidine chloride for 3 hours at  $25 \pm 2^\circ \text{C}$  before the record was made. Heavy time marks 5 seconds apart.

while 33 are being recorded at *D* (bottom)<sup>11</sup> Similar situations are also found in the heart

Fig 10 shows groups<sup>12</sup> of action currents separated by intervals of rest Each action current at *C* (top) passes over to *D* (bottom) but the forms of the curves are not identical in all cases

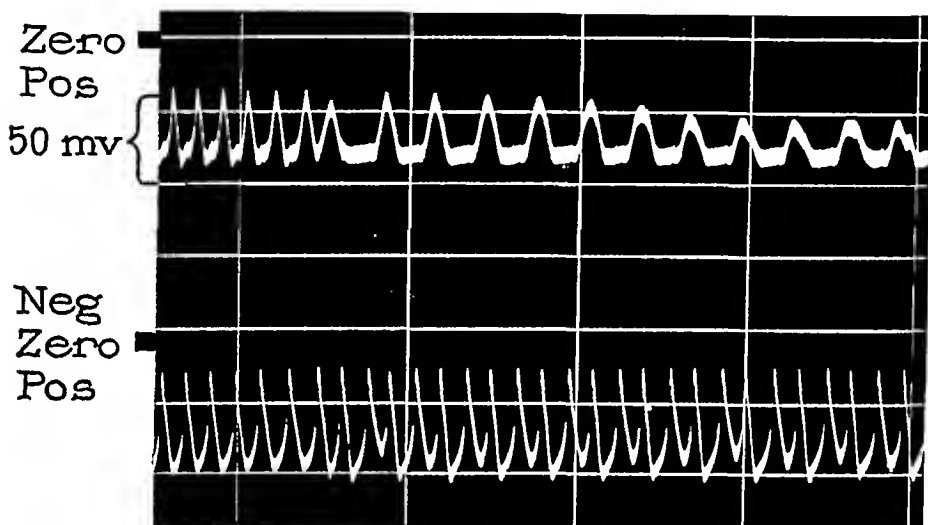


FIG 5 Shows the sudden appearance of a 2:1 ratio The first 7 action currents at *D* (bottom, cf Fig 1) are followed by action currents at *C* (top) but after this every other impulse registers at *C* and most of the time there is incomplete recovery of every other action current at *D*, i.e. the stimulus from the pacemaker arrives before recovery is finished and in these cases the action current does not register at *C*

Since *D* (bottom) moves first the pacemaker is at the right of *D*, probably at *F* which is in contact with 0.01 M KCl (the other spots are in contact with 0.01 M NaCl) The leads are 28 mm apart

The cell was freed from neighboring cells and kept for 40 days in Solution A at  $15 \pm 1^\circ\text{C}$  It was then placed in 0.01 M NaCl for 3 hours at  $25 \pm 2^\circ\text{C}$

Heavy time marks 5 seconds apart

In Fig 11 we see groups of action currents but the rhythms of *C* (top) and *D* (bottom) appear to be independent, indicating a com

<sup>11</sup> Whether *C* and *D* are acting independently is not evident

<sup>12</sup> Such groups are not uncommon in *Nitella* Cf Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1934-35, 18, 499 Hill, S E, and Osterhout, W J V, *J Gen Physiol*, 1938-39, 22, 91

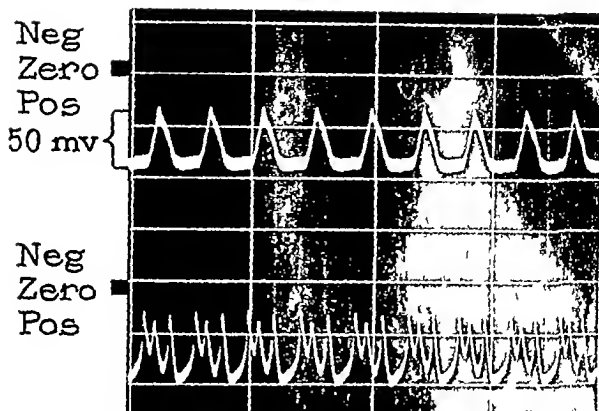


FIG 6 Shows a 3:1 ratio. Every third impulse passes over from *D* (bottom of Fig 1) to *C* (top). Since the bottom string *D* moves first the pacemaker is at the right of *D* and is probably at *F* which is in contact with 0.01 M KCl. The other spots are in contact with 0.01 M NaCl. The leads are 28 mm apart.

The cell was freed from neighboring cells and kept for 40 days in Solution A at  $15 \pm 1^\circ \text{C}$ . It was then placed for 3 hours in 0.01 M NaCl at  $25 \pm 2^\circ \text{C}$  before the record was made.

Heavy time marks 5 seconds apart.

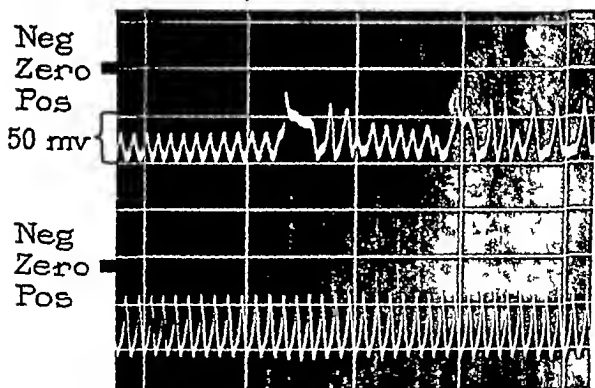


FIG 7 Here *D* (bottom of Fig 1) is regular and *C* (top) is partly irregular. Since *D* moves first the pacemaker is at the right of *D* probably at *F* which is in contact with 0.01 M KCl. *C* and *D* are in contact with 0.01 M NaCl. The leads are 28 mm apart.

The cell was freed from neighboring cells and kept for 40 days in Solution A at  $15 \pm 1^\circ \text{C}$ . It was then placed for 3 hours in 0.01 M NaCl at  $25 \pm 2^\circ \text{C}$  before making the record.

Heavy time marks 5 seconds apart.

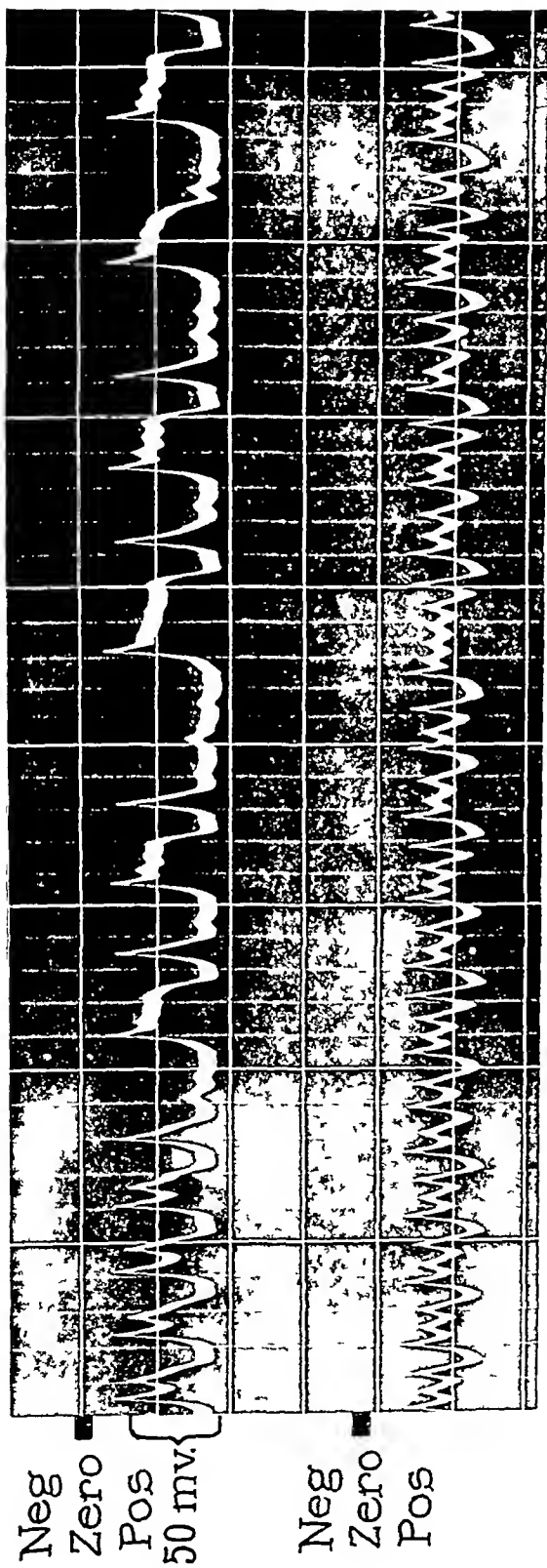


FIG. 8 Both *C* (top, cf Fig 1) and *D* (bottom) are irregular. The first part of the record appears to show that *D* moves first so that the pacemaker is at the right of *D*, probably at *F* which is in contact with 0.01 M KCl. *C* and *D* are in contact with 0.01 M NaCl. The leads are 28 mm apart.

The cell was freed from neighboring cells and kept for 41 days in Solution A at  $15 \pm 1^\circ\text{C}$ . It was then placed in 0.01 M NaCl for 2.5 hours at  $25 \pm 2^\circ\text{C}$  before the record was made.

Heavy time marks 5 seconds apart.

plete block between them. A striking instance of complete block is seen in Fig 12. The form of action curve seen at *C* (top) is common in cells treated<sup>13</sup> with NaCl but is seldom present in the same cell and at the same time as the quick action currents seen at *D* (bottom).

Groups of action currents and complete block are also found in

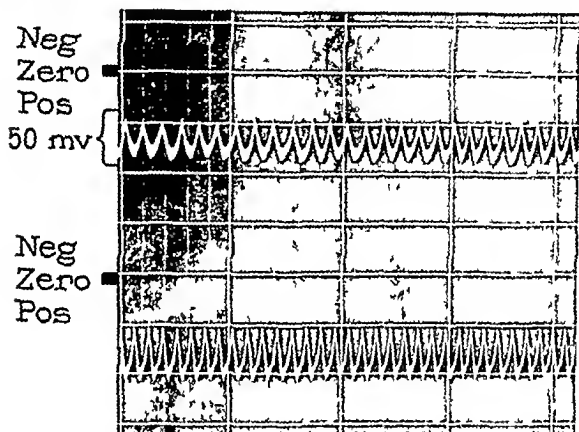


FIG 9 Here 33 action currents are recorded at *D* (bottom of Fig 1) while 26 are being recorded at *C* (top). As *F* was in contact with 0.01 M KCl it is probably the pacemaker for *D* (*C* and *D* are in contact with 0.01 M NaCl). The leads are 28 mm apart.

The cell was freed from neighboring cells and kept for 40 days in Solution A. It was then placed for 2.5 hours in 0.1 M NaCl at  $25 \pm 2^\circ \text{C}$  before the record was made.

Heavy time marks 5 seconds apart.

the heart.<sup>9, 10</sup> A great variety of other irregularities found in the heart may be duplicated in *Vitella*. One of these electrical alternans<sup>11</sup> is shown in Fig 13. Another shows groups in each of which the amplitude of the action curve first increases and then diminishes.<sup>14</sup> Still another is an analogue of auricular extra systole (Fig 14).

<sup>13</sup> Cf Hill S E and Osterhout W J V *J Gen Physiol*, 1938-39 22, 91  
Fig 13

<sup>14</sup> Cf Osterhout W J V and Hill S E *J Gen Physiol* 1934-35 18, 509

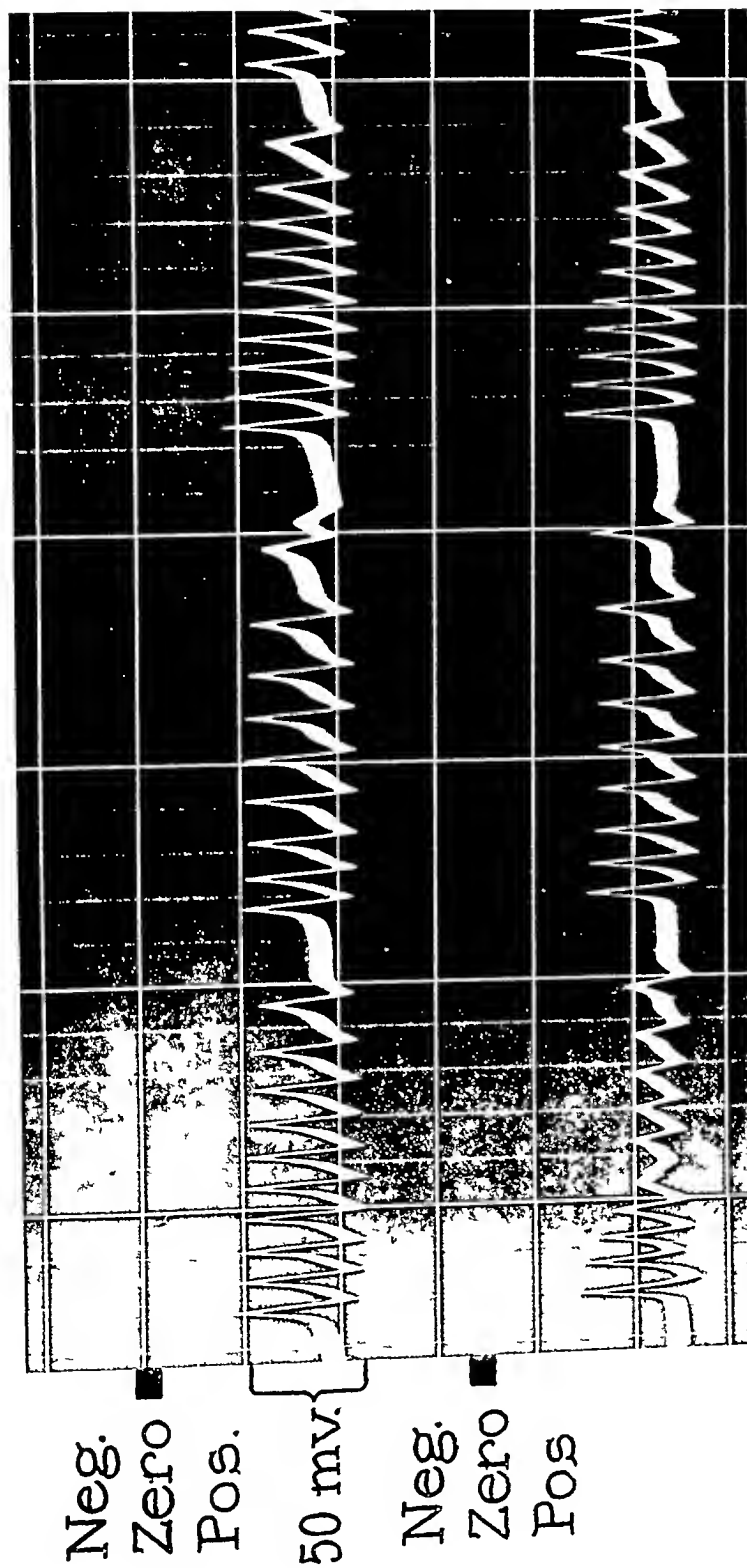


Fig 10 Shows groups of action currents with intervals of rest Since *C* (top, cf Fig 1) moves before *D* (bottom) the pacemaker is at the left of *C* (*C* and *D* are in contact with 0.01 M NaCl and *F* with 0.01 M KCl) The leads are 28 mm apart  
 The cell was freed from neighboring cells and kept for 41 days in Solution A at  $15 \pm 1^\circ\text{C}$  It was then placed in 0.01 M NaCl at  $25 \pm 2^\circ\text{C}$  for 2.5 hours before the record was made  
 Heavy time marks 5 seconds apart

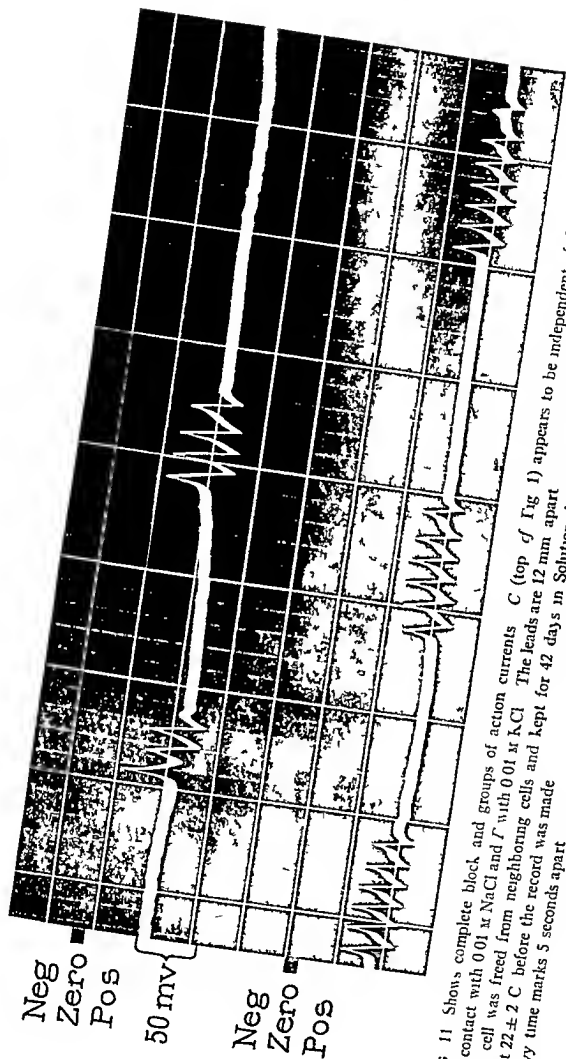


Fig 11 Shows complete block and groups of action currents C (top of Fig 1) appears to be independent of D (bottom) C and D are in contact with 0.01 M NaCl and  $r$  with 0.01 M KCl The leads are 12 mm apart The cell was freed from neighboring cells and kept for 42 days in Solution A at  $15 \pm 1^\circ \text{C}$  It was then placed for 3 hours in 0.01 M NaCl at  $22 \pm 2^\circ \text{C}$  before the record was made Heavy time marks 5 seconds apart



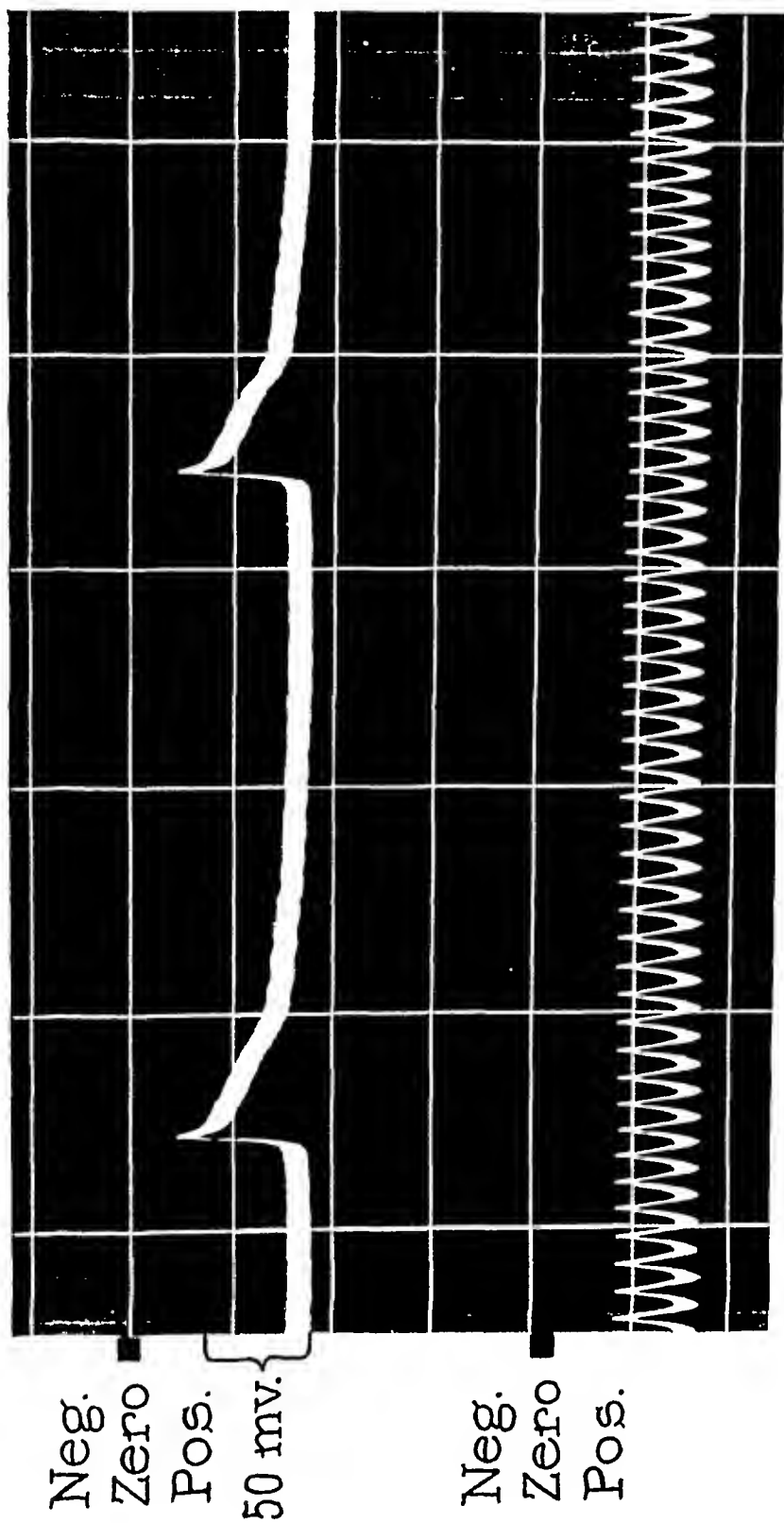


FIG 12 Shows complete block. *C* (top, cf Fig 1) and *D* (bottom) appear to be independent. The form of the action curve at *C* is one commonly found in cells treated with NaCl. *C* and *D* are in contact with 0.01 M NaCl, *F* with 0.01 M KCl. The leads are 28 mm apart.

The cell was freed from neighboring cells and kept for 40 days in Solution A at  $15 \pm 1^\circ\text{C}$ . It was then placed in 0.01 M NaCl for 3 hours at  $25 \pm 2^\circ\text{C}$  before the record was made.

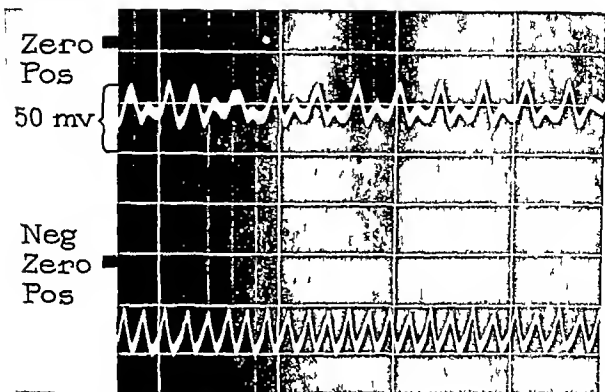


FIG 13 Shows 'electrical alternans' Each action current at *D* (bottom of Fig 1) is followed by one at *C* (top) where every other action curve is of smaller magnitude As *D* moves first the pacemaker is at the right of *D* probably at *F* which is in contact with 0.01 M KCl (*C* and *D* are in contact with 0.01 M NaCl) The leads are 18 mm apart

The cell was freed from neighboring cells and kept for 42 days in Solution A at  $15 \pm 1^\circ \text{C}$  It was then placed for 3 hours in 0.01 M NaCl at  $25 \pm 2^\circ \text{C}$  before the record was made

Heavy time marks 5 seconds apart



FIG 14 Shows changes like those observed in extra systole of the auricle i.e. an occasional premature impulse followed by an extra long pause (but not a pause of double length as in ventricular extra systole)

*D* (cf Fig 1) which is recorded is in contact with 0.01 M NaCl *F* is in contact with 0.01 M KCl and is apparently acting as pacemaker Whether there is a competition of pacemakers is not clear

The cell was freed from neighboring cells and kept for 30 days at  $15 \pm 1^\circ \text{C}$  in Solution A It was then placed for 3 hours in 0.01 M NaCl at  $22 \pm 2^\circ \text{C}$  before the record was made

Heavy time marks 5 seconds apart

Zero

Neg

Zero

Pos

Zero



FIG. 15 Shows a progressive change in the ability of C (top, cf Fig 1) to follow in detail the impulses coming from B<sub>2</sub> (bottom). The pacemaker is at the right of D, probably at  $t$ , which is in contact with 0.01 M KCl (the other spots were in contact with 0.01 M NaCl). The leads are 28 mm apart. The cell was freed from neighboring cells and kept for 30 days in Solution A at  $15 \pm 1^\circ\text{C}$ . It was then placed in 0.01 M NaCl for 5 hours at  $24 \pm 2^\circ\text{C}$  before the record was made. Heavy time marks 5 seconds apart.

The continuous record is cut into sections for purposes of reproduction.

Competition between pacemakers and shifts from one pacemaker to another which occur in the heart are also found in *Nitella* these will be described elsewhere<sup>15</sup>

#### DISCUSSION

It may seem surprising that so many of the irregularities found in the heart have analogies in *Nitella*. Evidently, two regions of a *Nitella* cell, only a short distance apart, can react differently and this appears to be the fundamental requisite for duplicating many of the differences of rhythm between auricle and ventricle.

Since the irregularities of conduction in *Nitella* so closely resemble those found in disorders of the heart it may be asked whether the cells of *Nitella* used in these experiments were in any way pathological. Regarding this it may be said that they were normal in appearance and that they remained so after a much longer exposure to Solution A, also that they remained normal in appearance after an exposure of several weeks to 0.01 M NaCl.

It may be added that in many cases the heart is quite normal in appearance when it shows the irregularities here discussed<sup>16</sup>. It therefore seems that similar irregularities can be brought about in the heart and in *Nitella* by certain conditions which do not produce abnormal appearances. To what extent these conditions are similar must be decided by future investigation.

In conclusion we may say that when recovery in a stimulated region is so slow that the region cannot respond normally to the next stimulus there may be arrhythmia and partial block. Much therefore depends on the rate of recovery. This may undergo a progressive change<sup>17</sup> as seems to be the case in Fig. 15<sup>17</sup>. As already indicated (p. 115) the rate of recovery may be greatly increased by exposure to certain salt solutions. It may be added that recovery can be (rever

<sup>15</sup> Regarding pacemakers see Auger, D. Comparaison entre la rythmicité des courants d'action cellulaires chez les végétaux et chez les animaux. Actualités sc. et indust. No. 314. Paris: Hermann et Cie, 1936.

<sup>16</sup> White, P. D., Heart disease. New York: The Macmillan Co., 2nd edition, 1937, pp. 610 ff.

<sup>17</sup> Sometimes the rate of recovery seems to slow down and then recover, cf. Hill, S. E. and Osterhout, W. J. V. *J. Gen. Physiol.* 1935-36, 19, 307.

sibly) suppressed, producing complete block. This appears to offer a promising field for further investigation.

The occurrence of closely similar phenomena in structures as different as the vertebrate heart and *Nitella* suggests that all tissues capable of conducting impulses are similar in certain fundamental properties and appears to justify the use of simple tissues to make clear the mechanism of more complex structures.

#### SUMMARY

Many forms of irregular rhythm and of partial block occurring in the vertebrate heart can be duplicated in *Nitella*.

In order to observe these phenomena the cells of *Nitella* are kept for 6 weeks or more in a nutrient solution. They are then exposed for 3 hours or less to 0.01 M NaCl, NaSCN, or guanidine chloride, which reduce the time required for the action current to about 1 second (the normal time is 15 to 30 seconds).

A pacemaker is established at one end of the cell by placing it in contact with 0.01 M KCl. This produces action currents at the rate of about 1 a second. Apparently some parts of the cell are unable to follow this rapid pace and hence fall into irregular rhythms (arrhythmia) and fail to register all the impulses (partial block).

We wish to express to A. E. Cohn and to A. G. Macleod our thanks for valuable criticisms.

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## EFFECT OF SODIUM SULFATE ON THE PHAGE BACTERIUM REACTION

By A P KRUEGER AND W L STRIETMANN\*

*(From the Department of Bacteriology, University of California, Berkeley)*

*(Accepted for publication, June 10, 1938)*

Two definite types of electrolyte effect on the phage bacterium reaction have been reported. Krueger and West (1) showed that minute concentrations of  $Mn^{++}$  accelerate lysis by depressing the lytic threshold,  $\tau e$ , by lowering the ratio of phage to bacteria requisite for lysis. Furthermore, the  $Mn$  ion shifts phage distribution, increasing the extracellular fraction to approximately four times the normal value. Due to the fact that less phage is required to lyse a given number of bacteria, the cellular phage producing mechanism is destroyed earlier in the reaction and there is a corresponding decrease in the total quantity of phage produced.

Scribner and Krueger (2) investigated a phenomenon first noted by Northrop, namely, that the addition of fairly large amounts of sodium chloride to the medium during the period of reaction between phage and susceptible organisms leads to an augmented end titre of phage. They concluded that 0.25 M NaCl raises the lytic threshold 5 to 10 fold. Therefore, the increased total yield is merely an expression of the fact that the bacteria are less susceptible to phage action and more phage must accumulate in the medium before lysis is initiated. Of particular significance with regard to the basic mechanism of phage formation is the further observation that 0.7 hour prior to the onset of lysis bacterial growth ceases whereas phage production continues at an undiminished rate. It would appear then that under some conditions bacterial growth is not an essential prerequisite for phage formation.

It is evident that variations in the concentration of certain electro-

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lytes can profoundly influence the kinetic relationships between the two reactants, phage and bacteria. We wish to present here the results of experiments in which bacteriophagy took place in the presence of sodium sulfate.

### Methods<sup>1</sup>

The symbols used are as follows:

[P] = concentration of phage activity units/ml

[B] = concentration of bacteria/ml

[P]<sub>0</sub> = initial concentration of phage/ml

[B]<sub>0</sub> = initial concentration of bacteria/ml

P U = phage activity units

1 The bacterial suspensions consisted of freshly harvested 16-hour cultures of *Staph. lococcus aureus* (strain  $S_2K$ ) grown on nutrient agar in Roux flasks and washed once in Locke's solution. Cell concentrations were determined by the centrifuged sediment method of Krueger (3). Broth was standard beef infusion containing 1 per cent Difco neopeptone, 0.5 per cent sodium chloride, and was adjusted to pH 7.4.

2 Phage titres were determined by the activity method of Krueger (4). The activity unit is the smallest amount of phage which will cause lysis when added to a certain number of susceptible cells under standard conditions. Our standard phage contains  $1 \times 10^{10}$  activity units. Because  $\text{Na}_2\text{SO}_4$  tends to slow down the phage-bacterium reaction its presence must be taken into account in titrating experimental solutions. We have made it a practice to titrate  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$  dilutions of all phage unknowns, these dilutions obviate any effect of  $\text{Na}_2\text{SO}_4$  on the titration system itself.

3 Determination of [bacteria]. As noted above the centrifuged sediment method was employed in preparing the living bacterial suspensions for each day's experiments. In the experimental series variations in [bacteria] were followed by three methods:

A. Turbidity readings were made directly on the sample or on an aliquot sufficiently diluted to come within the range of a formalized standard series (range  $5 \times 10^7$  to  $20 \times 10^7$  bacteria per ml).

B. Direct counts were used to check the turbidity measurements. Samples were diluted in gentian violet containing formalin and the numbers of cells were counted in the Hauser chamber.

C. Plate counts were made on broth cultures of  $S_2K$  containing no phage. Despite multiple sampling the plate count method was found to be inadequate for quantitative work.

<sup>1</sup> The experimental methods employed were essentially those reported in previous publications appearing for the most part in the *Journal of General Physiology*, 1929-38.

4  $\text{Na}_2\text{SO}_4$  effect Sterile  $\frac{M}{1}$   $\text{Na}_2\text{SO}_4$  was added to mixtures of phage and bacteria in broth in sufficient quantity to produce the desired final molarity. The mixtures were shaken in the  $36^\circ\text{C}$  water bath.

5 Rate of phage production To observe the effect of  $\text{Na}_2\text{SO}_4$  on the rate of phage production special concentrations of bacteria, phage and  $\text{Na}_2\text{SO}_4$  were used.  $[B]$  was  $3 \times 10^7$  bacteria per ml,  $[P]$  was  $1 \times 10^8$  P.U. per ml, and  $[\text{Na}_2\text{SO}_4]$  was  $\frac{M}{8}$ . These concentrations were selected to assure the reaction being completed within a reasonable length of time and to provide for adequate dilutions of phage unknowns so that there would be no possibility of  $\text{Na}_2\text{SO}_4$  or bacteria carried over from the reacting mixture affecting the titration system. The mixtures were made in broth at pH 7.4 and were maintained at  $36^\circ\text{C}$  in the water bath shaker. For total phage determinations samples were removed at intervals and were promptly diluted in broth kept at  $5^\circ\text{C}$ . The diluted samples from one experiment were accumulated and titrated together. For extracellular phage determinations samples of the reacting suspension were cooled in salt ice mixture and the bacteria were thrown down in the angle centrifuge. Dilutions of the supernatants were made in cold broth and were kept at  $5^\circ\text{C}$  until time for titration.

6 Lytic threshold experiments To detect the effect of  $\text{Na}_2\text{SO}_4$  on the lytic threshold of resting bacteria washed cells were grown in broth in the presence of  $\text{Na}_2\text{SO}_4$  and in its absence, using an initial concentration of  $1 \times 10^7$  cells per ml. When [bacteria] reached  $6 \times 10^8$  cells per ml growth was stopped by using the suspensions and mixtures containing a final concentration of  $6 \times 10^7$  bacteria per ml and varying amounts of phage were prepared. The mixtures were placed in the water bath shaker at  $36^\circ\text{C}$  and bacterial concentrations were determined at 0.2 hour intervals. The lowest ratio of phage to bacteria giving lysis without bacterial growth was selected as the critical value.

## RESULTS

When the phage bacterium reaction takes place in the presence of increasing concentrations of sodium sulfate two results are outstanding. First, the time of lysis is prolonged and second, the end titre of the lysate is increased. As shown in Table I the increase in time of lysis is roughly proportional to the salt content. The end titre, however, reaches a maximum in  $\frac{M}{8}$   $\text{Na}_2\text{SO}_4$  and further increase in salt concentration, while delaying the time of lysis does not affect the titre of the lysate.

A comparison of the phage production curves in Figs. 1 and 2 shows that there is quite a lag phase in the presence of sodium sulfate.

In one hour the salt-containing suspension has formed  $4 \times 10^8$  P U / ml while the control mixture has produced  $1.0 \times 10^{10}$  P U / ml. From 1 hour on the rate of phage production in the presence of  $\text{Na}_2\text{SO}_4$  becomes logarithmic with time but the rate of increase is only 40-fold per hour as contrasted with the control rate of a 125-fold increase per hour.

Phage distribution between cells and medium likewise is altered by  $\text{Na}_2\text{SO}_4$ . By 0.5 hour in the control 80 per cent of the phage is in or on the cells while the corresponding figure for the intracellular fraction in the salt mixture is 40 per cent. At 1.0 hour 50 per cent of

TABLE I

*Time of Lysis and Total Phage Produced in Broth Containing Various Concentrations of  $\text{Na}_2\text{SO}_4$*

The mixtures were each prepared in a total volume of 10 ml of broth and the tubes were shaken at  $36^\circ\text{C}$

Molarity of $\text{Na}_2\text{SO}_4$	[P] <sub>0</sub>	[B] <sub>0</sub>	Lysis	Total phage formed
			hrs	
0.05	$1 \times 10^7$	$3 \times 10^7$	2.50	$7 \times 10^{10}$
0.075	"	"	2.70	$1 \times 10^{11}$
0.10	"	"	3.20	$1.5 \times 10^{11}$
0.125	"	"	3.60	$3.0 \times 10^{11}$
0.150	"	"	3.97	$3.0 \times 10^{11}$
0.175	"	"	4.74	$3.0 \times 10^{11}$
0.200	"	"	6.00	$3.0 \times 10^{11}$
0.250	"	"	No lysis	
Control	"	"	2.10	$2 \times 10^{10}$

the total phage still remains extracellular in the  $\frac{\text{M}}{8}$   $\text{Na}_2\text{SO}_4$  suspension.

Casual inspection of Figs 1 and 2 shows that the lytic threshold for actively growing bacteria is raised by  $\text{Na}_2\text{SO}_4$ . In the control set (Fig 1) the critical value is 80 P U per bacterium whereas in the salt mixture the threshold rises to 310 P U per bacterium (Fig 2). Tests carried out with resting cells likewise showed the threshold to be four times greater under the influence of  $\frac{\text{M}}{8}$   $\text{Na}_2\text{SO}_4$  (Table II).

Another point of interest in the phage bacterium reaction occurring in  $\frac{M}{8}$   $\text{Na}_2\text{SO}_4$  broth is the long period just before lysis during which bacterial growth ceases while phage production continues. The

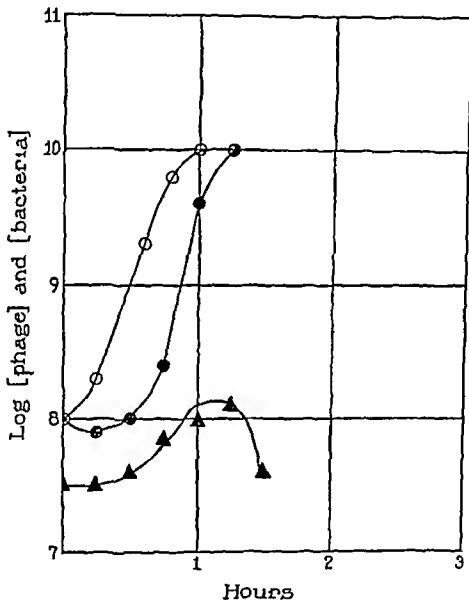


FIG 1 The growth of bacteria and phage production in plain broth  $[B] = 3 \times 10^7$  bacteria/ml  $[P]_0 = 1 \times 10^8$  P U/ml,  $\circ$  = total phage in activity units/ml,  $\bullet$  = extracellular phage in activity units/ml,  $\blacktriangle$  = bacteria/ml,  $36^\circ\text{C}$ , pH 7.4

bacterial growth curves were followed by means of turbidity measurements, direct microscopic counts, and in the case of suspensions containing no phage by means of plate counts. Evidently  $\frac{M}{8}$   $\text{Na}_2\text{SO}_4$  has

no effect on bacterial growth either in the presence or in the absence of phage except for the production of the prelytic plateau noted above

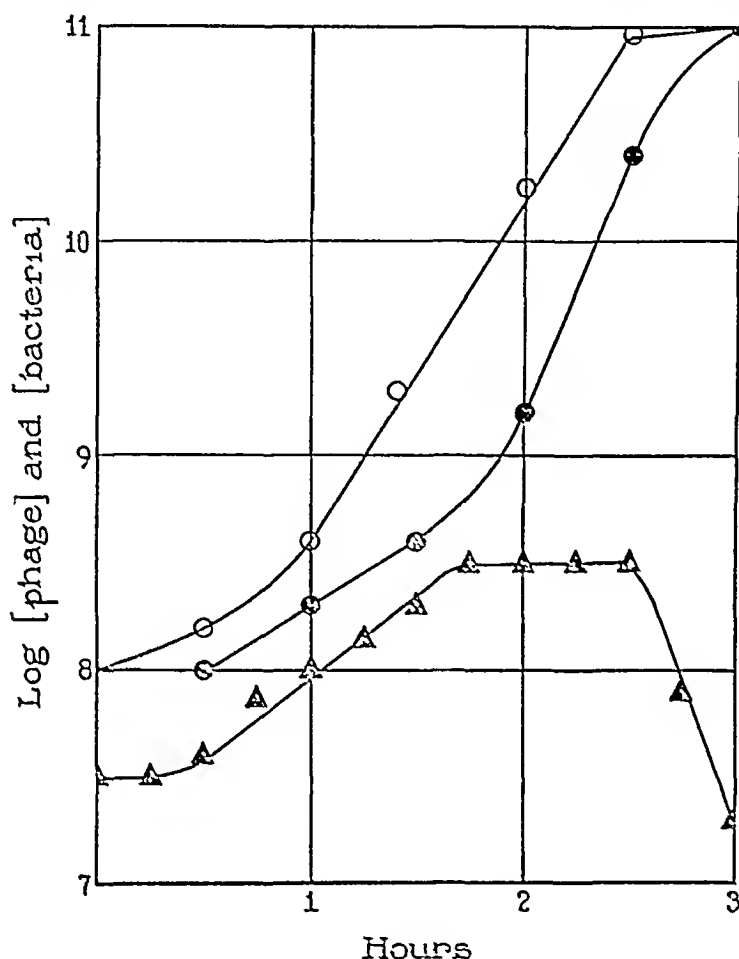


FIG 2 The growth of bacteria and phage production in  $\frac{M}{8}$   $\text{Na}_2\text{SO}_4$  broth  
 $[B]_0 = 3 \times 10^7$  bacteria/ml,  $[P]_0 = 1 \times 10^5$  P U/ml,  $\circ$  = total phage in activity units/ml,  $\bullet$  = extracellular phage in activity units/ml,  $\blacktriangle$  = bacteria/ml,  
 $[\text{Na}_2\text{SO}_4] = \frac{M}{8}$   $36^\circ\text{C}$ , pH 7.4

The influence of  $\text{Na}_2\text{SO}_4$  on distribution can be demonstrated in another way. Mixtures containing  $5 \times 10^5$  bacteria/ml and  $1 \times 10^5$  P U / ml in broth with and without  $\frac{M}{8}$   $\text{Na}_2\text{SO}_4$  are held at  $5^\circ\text{C}$ . At intervals samples are removed the resting cells centrifuged down,

and the supernatants titrated. The suspension containing  $\text{Na}_2\text{SO}_4$  comes to equilibrium with 40 per cent of the phage outside the cells, in the control mixture 10 per cent of the phage is extracellular.

TABLE II

*Effect of  $\text{Na}_2\text{SO}_4$  on the Lytic Threshold of Resting Cells. Determination of Critical Phage/Bacteria Ratio for Lysis in Absence of Bacterial Growth. Staphylococcus Phage Mixtures with [Bacteria] Constant ( $[B] = 6 \times 10^7$  Bacteria/ml) and [Phage] Varying in Plain Broth and in Broth*

Containing  $\frac{M}{8} \text{Na}_2\text{SO}_4$

The bacterial suspensions consisted of young cells. Growth was inhibited by preliminary icing. After the mixtures were made they were placed in the water bath shaker at  $36^\circ\text{C}$  and turbidity readings were made every 0.2 hr. Multiply figures in columns by  $10^7$ .

The critical phage/bacteria ratio in plain broth is 83 P.U./bacterium. In  $\frac{M}{8} \text{Na}_2\text{SO}_4$  it is 330 P.U./bacterium.

Standard phage $1 \times 10^8$ P.U./ml. or $\text{Na}_2\text{SO}_4$ phage $1 \times 10^{10}$ P.U./ml	8		7		6		5		4		3		2		1	
Broth	1		2		3		4		5		6		7		8	
Bacteria $6 \times 10^7$ /ml	1		1		1		1		1		1		1		1	
Time	Control		Control		Control		Control		Control		Control		Control		Control	
	$\text{Na}_2\text{SO}_4$		$\text{Na}_2\text{SO}_4$		$\text{Na}_2\text{SO}_4$		$\text{Na}_2\text{SO}_4$		$\text{Na}_2\text{SO}_4$		$\text{Na}_2\text{SO}_4$		$\text{Na}_2\text{SO}_4$		$\text{Na}_2\text{SO}_4$	
hrs																
0.2	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
0.4	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
0.6	3	6	3	6	3	6	3	6	8	6	8	6	8	6	8	8
0.8	0	3	0	3	0	3	0	3	6	3	8	3	10	3	10	8
1.0		0		0		0		0	0	0	4	0	8	0	8	4
1.2											0		4		4	0
1.4													0		0	

## DISCUSSION

The reaction between *Staphylococcus aureus* and the homologous bacteriophage in the presence of  $\text{Na}_2\text{SO}_4$  has several interesting aspects. In the first place bacterial growth shows no departure from the normal. Phage production, however, has a rather long lag phase after which it proceeds logarithmically with time but at only one

third the normal rate. Apparently it is the action of  $\frac{M}{8}$   $\text{Na}_2\text{SO}_4$  in altering phage distribution which is responsible for both the long lag phase and the lower rate of phage production. Somehow the salt changes the bacterium's surface so that much less than the usual amount of phage is taken up. The phage initially present in the mixture is thus kept from optimal contact with the phage precursor in the actively growing cells and the phage-forming mechanism consequently functions at a slower pace.

$\frac{M}{8}$   $\text{Na}_2\text{SO}_4$  has a marked influence on the susceptibility to phage action of both resting and growing bacterial cells. It takes four times the normal amount of phage to lyse a bacterium in the presence of this concentration of sodium sulfate. This phage resistance is attained without observable destruction of the phage-forming system and it is because of the increased resistance that the bacteria are enabled to continue manufacturing phage with the eventual result that from ten to twenty times the customary phage yield is attained.

#### SUMMARY AND CONCLUSIONS

Bacteriophagy taking place in the presence of  $\frac{M}{8}$   $\text{Na}_2\text{SO}_4$  has the following pronounced characteristics:

- A Time of lysis is considerably prolonged
- B The bacteria take up less than the normal amount of phage
- C Phage production occurs at one-third the customary rate
- D It takes four times as much phage to lyse a  $\text{Na}_2\text{SO}_4$ -treated bacterium than a normal one
- E Bacterial growth is not affected by  $\text{Na}_2\text{SO}_4$

The lag phase and the lowered rate of phage production can be attributed to the  $\text{Na}_2\text{SO}_4$  effect on the cell surface. Less phage is taken up by the cells and contact of phage with the bacterium's precursor-producing mechanism is impeded.

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# CALCULATIONS OF BIOELECTRIC POTENTIALS

## IV SOME EFFECTS OF CALCIUM ON POTENTIALS IN NITELLA

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The action of calcium on the P D of *Nitella* is of especial interest in certain cases where it modifies the effect of potassium

In some cells<sup>1</sup> the negative change produced by substituting 0.01 M KCl for 0.01 M NaCl is not altered by adding to the KCl solution enough CaCl<sub>2</sub> to make its concentration 0.005 M. But in other cases calcium has more effect.

In a previous paper<sup>2</sup> the values of  $\mu_K$  and  $\mu_{Na}$ , the apparent mobilities of K<sup>+</sup> and Na<sup>+</sup> in  $\bar{Y}$ , the outer non aqueous protoplasmic surface layer, were calculated as  $\mu_K = 11.9$  and  $\mu_{Na} = 7.93$ . Using these values we may evaluate the effect of calcium by employing Henderson's equation

This may be written<sup>3</sup> (for 20°C)

$$P.D. = 58 \frac{(U_I - V_I) - (U_{II} - V_{II})}{(U_I' + V_I') - (U_{II}' + V_{II}')} \log_{10} \frac{U_I' + V_I'}{U_{II}' + V_{II}'} \quad (1)$$

in which  $U_I = \mu_1 c_1 + u c_2$

$V_I = v_1 c_1 + v_2 c_2$

$U_I' = \mu_1 w_1 c_1 + \mu_2 w_2 c_2$

$V_I' = v_1 w_1 c_1 + v_2 w_2 c_2$

where  $c$  = concentration of cation and  $\bar{c}$  of anion

$\mu$  = mobility of cation and  $v$  of anion

$w$  = valence of cation and  $\bar{w}$  of anion

<sup>1</sup> This is more apt to be true of freshly collected cells than of those which have been freed from neighboring cells and kept for some weeks in the laboratory in Solution A at 15 ± 1°C. Regarding Solution A see Osterhout W J V, and Hill S E *J Gen Physiol* 1933-34, 17, 87.

<sup>2</sup> Hill, S E and Osterhout W J V *Proc Nat Acad Sc* 1938 24, 312.

<sup>3</sup> Cf. Michaelis L, Hydrogen ion concentration, Baltimore, The Williams and Wilkins Co. 1926 179. This formula is incorrectly given in some text books.



The subscripts I and II refer to the two solutions, the subscripts 1 and 2 to the different ions. All values relate to  $X$ , the outer non-aqueous protoplasmic surface layer.

In the case of 0.005 M *vs* 0.0005 M  $\text{CaCl}_2$  it has been pointed out by Longworth<sup>4</sup> that when we put  $C_{\text{Cl}} = 2 C_{\text{Ca}}$  this equation reduces to

$$P.D. = 58 \frac{u_{\text{Ca}} - 2v_{\text{Cl}}}{2u_{\text{Ca}} + 2v_{\text{Cl}}} \log_{10} \frac{c'_{\text{Ca}}}{c''_{\text{Ca}}} \quad (2)$$

where  $c'$  relates to Solution I (0.005 M  $\text{CaCl}_2$ ) and  $c''$  to Solution II (0.0005 M  $\text{CaCl}_2$ ). The sign of the calculated P.D. is that of Solution II in the external circuit.

It is also pointed out by Longworth that equation (2) can be derived by starting with the general equation<sup>5</sup>

$$E_L = \frac{RT}{F} \int \sum \frac{t_i}{z_i} d \ln a_i \quad (3)$$

in which  $t$  is the transference number and  $z$  is the valence of the  $i$ th ion, account being taken of its sign. In the case of  $\text{CaCl}_2$ , assuming a constant transference number and activities equal to concentrations the equation becomes

$$E_L = \frac{RT}{F} \left( \frac{t_{\text{Ca}}}{2} \right) \ln_e \frac{c'_{\text{Ca}}}{c''_{\text{Ca}}} - \frac{RT}{F} (t_{\text{Cl}}) \ln_e \frac{c'_{\text{Cl}}}{c''_{\text{Cl}}} \quad (4)$$

But  $t_{\text{Ca}} + t_{\text{Cl}} = 1$  and although  $c_{\text{Cl}} > c_{\text{Ca}}$  we have

$$\frac{c'_{\text{Ca}}}{c''_{\text{Ca}}} = \frac{c'_{\text{Cl}}}{c''_{\text{Cl}}} = \frac{c'}{c''} \quad (5)$$

$$E_L = \frac{RT}{F} \left( \frac{t_{\text{Ca}}}{2} \right) \ln_e \frac{c'}{c''} - \frac{RT}{F} (1 - t_{\text{Ca}}) \ln_e \frac{c'}{c''} \quad (6)$$

$$= \frac{RT}{F} \left( \frac{3t_{\text{Ca}}}{2} - 1 \right) \ln_e \frac{c'}{c''} \quad (7)$$

$$= \frac{RT}{F} \left( \frac{u_{\text{Ca}} - 2v_{\text{Cl}}}{2u_{\text{Ca}} + 2v_{\text{Cl}}} \right) \ln_e \frac{c'}{c''} \quad (8)$$

<sup>4</sup> Longworth, L. G., personal communication.

<sup>5</sup> Cf. MacInnes, D. A., and Longworth, L. G., The potentials of galvanic cells with liquid junctions, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1936, 4, 18.

At 20°C this may be written

$$P.D. = 58 \left( \frac{u_{Ca} - 2v_{Cl}}{2u_{Ca} + 2v_{Cl}} \right) \log_{10} \frac{c'}{c''} \quad (9)$$

$$= 58 \frac{\frac{u_{Ca}}{+} - \frac{v_{Cl}}{-}}{u_{Ca} + v_{Cl}} \log_{10} \frac{c'}{c''} \quad (10)$$

where  $z^+$  and  $z^-$  are the valencies of the cation and anion respectively (it should be pointed out that in all the equations  $u_{Ca}$  relates to  $\frac{Ca^{++}}{2}$  and not to  $Ca^{++}$ , i.e., it is the mobility per equivalent of calcium ion and not per mole

Similarly for  $Na_2SO_4$  we have

$$P.D. = 58 \frac{2u_{Na} - v_{SO}}{2u_{Na} + 2v_{SO}} \log_{10} \frac{c'}{c''} \quad (11)$$

Equations (9) and (10) are identical with equation (2)

No matter how high we put  $u_{Ca}$  we find that with  $c'_{Ca} + c''_{Ca} = 10$  the calculated value of the P.D. does not exceed 29 mv if the partition coefficient remains constant. But if the partition coefficient  $S_{CaCl_2}$  ( $\approx$  concentration in  $\lambda$  + concentration in the external solution) increases with concentration (as happens with certain salts in models<sup>6</sup>) so that when  $c'_{Ca} + c''_{Ca} = 10$  in the external solution we have in  $\lambda$   $c'_{Ca} + c''_{Ca} = 19.4$  the calculation gives  $u_{Ca} = 20$  for a P.D. of 32 mv (as reported<sup>7</sup> in Table I)

As the value of  $u_{Ca}$  depends on that assigned to  $S_{CaCl_2}$ , its significance is doubtful but the following calculations may be of interest. It has been shown previously<sup>8</sup> that from the data in Table I we get  $u_K = 11.9$  and  $u_{Na} = 7.93$  if the value of  $S_{KCl} + S_{NaCl}$  is taken as 60 we get the observed value of 95 mv for 0.01 M KCl vs 0.01 M NaCl. Taking  $u_{Ca}$  as 20 we must put  $S_{KCl} + S_{CaCl_2}$  as 27.5 to get the observed value of 62 mv given in Table I for 0.01 M KCl vs 0.005 M  $CaCl_2$ .

The value of the partition coefficient probably increases with concentration as already assumed in the case of  $CaCl_2$ . If this increase is more rapid for KCl than for NaCl it would explain why the potassium effect falls off with dilution below 0.01 M.

Since  $S_{KCl} + S_{CaCl_2}$  is taken as 27.5 and  $S_{KCl} + S_{NaCl}$  as 60 the value of  $S_{NaCl} + S_{CaCl_2}$  may be taken as  $27.5 + 60 = 87.5$ . Calculation on this basis gives for the

<sup>6</sup> Osterhout, W. J. V., Kamerling, S. E. and Stanley, W. M., *J. Gen. Physiol.*, 1933-34, 17, 469.

<sup>7</sup> The experiments were performed on *Nitella flexilis*, Ag., using the technique described in a former paper (cf. Hill, S. E. and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 541). Temperature  $20 \pm 3^\circ C$ . All measurements of P.D. were made from photographic records.

P D of 0.01 M NaCl vs 0.005 M  $\text{CaCl}_2$ <sup>8</sup> 14 mv instead of the observed 22 mv (if we put  $S_{\text{NaCl}} - S_{\text{CaCl}_2} = 0.25$  we get 22 mv) This may indicate that  $\text{CaCl}_2$  changes the non-aqueous surface layer as it diffuses through it and comes in contact with the NaCl already present

TABLE I

The sign \* of the P D is positive unless otherwise stated and is that of the first mentioned solution as observed in the external circuit (In all cases the change was made in both directions, e.g. from dilute to concentrated and *vice versa* or from NaCl to KCl and *vice versa*)

Solution	P D	No of observations	No of cells	Calculated values
Concentration potentials (homoionic junctions)				
0.001 M KCl vs 0.01 M KCl	49 $\pm$ 0.49	95	41	—
0.001 M NaCl vs 0.01 M NaCl	45 $\pm$ 0.69	75	37	—
0.0005 M $\text{CaCl}_2$ vs 0.005 M $\text{CaCl}_2$	32 $\pm$ 0.65	60	31	—
0.001 M KCl + 0.0005 M $\text{CaCl}_2$ vs 0.01 M KCl + 0.005 M $\text{CaCl}_2$	45 $\pm$ 1.12	75	41	43
0.001 M NaCl + 0.0005 M $\text{CaCl}_2$ vs 0.01 M NaCl + 0.005 M $\text{CaCl}_2$	33 $\pm$ 0.73	70	37	28
Chemical potentials (heteroionic junctions)				
0.01 M KCl vs 0.01 M NaCl	-95 $\pm$ 2.0	120	44	—
† 0.01 M KCl vs 0.01 M KCl + 0.005 M $\text{CaCl}_2$	-30 $\pm$ 2.2	100	44	—
0.01 M KCl vs 0.005 M $\text{CaCl}_2$	-62 $\pm$ 2.6	38	28	—
0.01 M KCl + 0.005 M $\text{CaCl}_2$ vs 0.01 M NaCl + 0.005 M $\text{CaCl}_2$	-42 $\pm$ 2.3	75	40	-28
0.01 M NaCl vs 0.005 M $\text{CaCl}_2$	22 $\pm$ 1.2	40	31	14

\* The P D is called positive when positive current tends to flow outward from the sap across the protoplasm to the external solution

† Very small values have not been considered (in many cases the value was zero)

According to Henderson's equation the addition of solid  $\text{CaCl}_2$ , without change of mobilities or of partition coefficients, to a solution of KCl should make it more negative but in reality it becomes more positive. The calculation shows that if the ratio of partition co-

<sup>8</sup> Here  $\text{CaCl}_2$  is negative to NaCl

efficients is  $S_{KCl} - S_{CaCl_2} = 27.5$  then 0.01 M KCl should be positive to 0.01 M KCl + 0.005 M  $CaCl_2$  by 0.6 mv. But it is really negative by 30 mv, as shown in Table I. This would result if  $\mu_K$  or  $S_{KCl}$  were lowered considerably by the action of  $CaCl_2$ .

If  $\mu_K$  were lowered considerably the concentration effect would fall off greatly but this is not the case. The concentration effect of KCl for a 10 fold dilution is 49 mv and for KCl +  $CaCl_2$  is 45 mv (Table I) which would indicate that  $\mu_K$  is about the same in both cases. Probably the simplest assumption is that in the presence of calcium the value of  $S_{KCl}$  falls off. If in the mixture of KCl +  $CaCl_2$  the ratio  $S_{KCl} - S_{CaCl_2}$  fell to 5.4 the calculation would give<sup>9</sup> for 0.01 M KCl vs 0.01 M KCl + 0.005 M  $CaCl_2$  a p.d. of 30 mv with the mixture<sup>10</sup> positive to pure KCl. This agrees with observation (Table I).

It might be suggested that calcium in this case merely has its ordinary antagonistic effect. But it takes very little calcium to form an ordinary balanced solution (e.g. 0.01 M KCl + 0.0005 M  $CaCl_2$ ) which preserves the life of the cell indefinitely. To affect the p.d. in the manner described in this paper much more calcium is necessary (usually about 10 times as much). It is a striking fact that when we employ 0.1 M instead of 0.01 M KCl<sup>11</sup> the addition of 0.05 M  $CaCl_2$  usually has little or no effect on the p.d.

Moreover with certain cells the addition of as much as 0.005 M calcium to 0.01 M KCl has little or no effect on the p.d. although the normal antagonistic action of calcium is seen when its concentration is only 0.0005 M.

Another possibility is that calcium acts by precipitating an organic substance (or group of substances), called for convenience  $R_p$ , which sensitizes the cell to the action of potassium. If, as suggested in a

<sup>9</sup> Presumably this value varies with conditions like that of other partition coefficient ratios.

<sup>10</sup> For purposes of calculation we assume that in the non aqueous protoplasmic surface we have in arbitrary units for pure KCl  $c_{KCl} = 55$  and for the mixture  $c_{KCl} = 10.8$  and  $c_{CaCl_2} = 1$ .

<sup>11</sup> In short experiments the effects of 0.1 M KCl are fully reversible in the absence of calcium.

<sup>12</sup> To alter the effect of lower concentrations of KCl (0.01 M to 0.05 M) we need  $CaCl_2$  of the same normality as KCl less than this usually has little or no effect.

former paper,<sup>13</sup> the solubility of this substance is decreased by calcium we may expect that as the concentration of calcium increases beyond a certain point the surface will become less sensitive to the action of potassium (unless the latter has too high a concentration<sup>14</sup> as appears to be the case with 0.1 M KCl)

Hober<sup>15</sup> has suggested that in muscle calcium influences the colloidal structure in such fashion as to diminish the effect of potassium on the P D

As the result of the foregoing we arrive at the following values  $n_K = 11.9$ ,  $n_{Na} = 7.93$ ,  $n_{Ca} = 20$ . The ratios of partition coefficients are,  $S_{KCl} - S_{NaCl} = 60$ ,  $S_{KCl} - S_{CaCl_2} = 27.5$ ,  $S_{NaCl} - S_{CaCl_2} = 0.46$  in a mixture of KCl + CaCl<sub>2</sub> we have  $S_{KCl} - S_{CaCl_2} = 5.4$  and in a mixture of NaCl + CaCl<sub>2</sub> we have  $S_{NaCl} - S_{CaCl_2} = 0.46$

Using these values we obtain the calculated values shown in Table I. For the concentration effect the agreement between calculated and observed values is very satisfactory for the chemical effect it is less so but here we must allow for complicating factors

Let us now consider another effect of calcium. Fig. 1 shows that when the concentration of a mixture of KCl + CaCl<sub>2</sub> increases beyond a certain point an action current is encountered after which the level of the curve is higher by a definite amount. This is also found in pure KCl. The latter has been described in a previous paper<sup>16</sup> and as the behavior of the mixture is closely similar it need not be discussed here in detail. The effect of calcium is to raise the concentration at which the action current occurs. This is evident on comparing Fig. 1 with the corresponding figure in the previous paper.<sup>16</sup>

Still another effect of calcium is to inhibit the production of rapid action currents when cells are exposed to 0.01 M NaCl, as described elsewhere.<sup>17</sup>

<sup>13</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 105

<sup>14</sup> A high concentration of potassium would tend to increase the concentration of  $R_p$  if, as suggested in a former paper (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 107), the latter is a compound of potassium

<sup>15</sup> Hober, R., *Arch. ges. Physiol.*, 1904-05, 106, 599, 1916-17, 166, 582, 1929, 222, 82

<sup>16</sup> Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 541

<sup>17</sup> Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 91

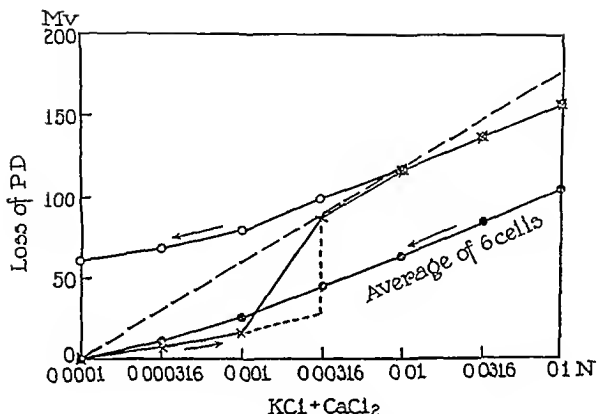


FIG 1 Changes of PD produced by a mixture of equal parts of a solution of KCl and a solution of  $\text{CaCl}_2$  of the same normality. The concentrations give the normality of each salt in the mixture, thus 0.1 N signifies a solution containing 0.1 N KCl + 0.1 N  $\text{CaCl}_2$ .

The broken straight line approximates the theoretical slope of the curve showing change of diffusion potential when the concentration of KCl in contact with *Nitella* increases and the mobility of  $\text{K}^+$  greatly exceeds that of  $\text{Cl}^-$ , partition coefficients being constant. The scale of abscissae is logarithmic; each step is made by multiplying by 3.16 ( $= 10^{0.5}$ ).

The curve with crosses shows measurements on a single cell as the concentration increases (arrows pointing upward). The curve with open circles shows measurements on the same cell as the concentration decreases (arrows pointing downward). The lowest curve shows the average of 6 cells as the concentration of KCl decreases.

The slopes of the curves do not exceed the theoretical except in the curve with crosses where 0.001 is replaced by 0.00316 N. At this point the change in PD occurs in two steps as indicated by the dotted line. The first step does not exceed the theoretical, the second is larger and is due to an action current which permanently raises the level of the curve.

In conclusion we may say that in these cells the mobilities of  $\text{K}^+$  and  $\text{Na}^+$  were much closer together than in certain cells studied earlier<sup>18</sup>.

<sup>18</sup> Osterhout, W. J. V. *J. Gen. Physiol.* 1929-30 13, 715.

(the values are 11.9 and 7.93 respectively instead of 85.45 and 2.18) It is possible that this is due in part to the treatment in the laboratory.

We know that mobilities can be influenced in various ways. For example, guaricol greatly lessens the mobility of  $K^+$  in *Valonia*<sup>19</sup> and increases that of  $Na^+$  in both *Valonia* and *Halocystis*.<sup>20</sup> Treatment with distilled water greatly lowers the mobility of  $K^+$  in *Nitella*.<sup>21</sup>

We also see that in these *Nitella* cells the partition coefficients differ from those found in earlier studies. For example  $S_{KCl} - S_{NaCl}$  is 60 instead of unity. We find that calcium appears to reduce  $S_{KCl}$  from 27.5 to 5.4. This recalls experiments which show that distilled water may lessen the partition coefficient of  $KCl$ .<sup>22</sup>

We may conclude that mobilities and partition coefficients are variable and can be brought, to some extent at least, under experimental control.

#### SUMMARY

In *Nitella* the substitution of  $KCl$  for  $NaCl$  changes the P.D. in a negative direction. In some cases this change is lessened by adding solid  $CaCl_2$  to the solution of  $KCl$ . This may be due to lessening the partition coefficient of  $KCl$  or to decreasing the solubility of an organic substance which sensitizes the cell to the action of  $KCl$ .

Little or no correlation exists between this effect of calcium and its ordinary antagonistic action in producing a balanced solution which preserves the life of the cell indefinitely.

$CaCl_2$  is negative to  $NaCl$  but positive to  $KCl$ .

The effects of mixtures of  $KCl$ ,  $NaCl$ , and  $CaCl_2$  are discussed. The concentration effect of a mixture of  $KCl + CaCl_2$  shows certain peculiarities due to action currents; these resemble those found with pure  $KCl$ .

These studies and others on *Nitella*, *Valonia*, and *Halocystis* indicate that mobilities and partition coefficients are variable and can be brought under experimental control.

<sup>19</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, 20, 13.

<sup>20</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 707.

<sup>21</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, 18, 987.

<sup>22</sup> Osterhout, W. J. V., and Hill, S. E., *Proc. Nat. Acad. Sc.*, 1938, 24, 427.

## THE KINETICS OF PENETRATION

### XV THE RESTRICTION OF THE CELLULOSE WALL

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The object of the present paper is to show the effect of the cellulose wall on the rate of entrance of electrolytes and of water into *Valonia macrophysa*, Kutz

In the laboratory, when the cells are illuminated, the rate of growth in sea water is slow (between 0.5 to 1.0 per cent per day), and this slow growth appears to limit in some way the rate of entrance of electrolytes since the osmotic pressure and the halide concentration of the sap are maintained at approximately constant levels

If the cell is impaled on a capillary so that space is provided into which the vacuolar sap can move, the restriction due to the cell wall is abolished and under these conditions we should expect the rate of entrance of water and of electrolyte to increase

It will be shown that this prediction is realized the rate of entrance of both water and electrolyte is increased 15 fold on the average

In the case of *Halocystis* cells impaled on a capillary for electrical measurements the rise of liquid in the capillary has been observed by Blinks,<sup>1</sup> who states<sup>2</sup> that it may continue indefinitely. He did not, however, study the phenomenon systematically. The present experiments are an outgrowth of Blinks' observations

Impaled *Valonia* cells have been used by Kornmann<sup>3</sup> as osmometers in hypertonic solutions containing alcohols and salts, but his results have no bearing on the present work

<sup>1</sup> Blinks, L. R., *J. Gen. Physiol.* 1934-35 18, 409

Private communication

<sup>3</sup> Kornmann P. *Protoplasma* 1934 21, 340 1935 23, 34



## EXPERIMENTAL

Since *Valonia* cells were first impaled on capillaries in order to measure potential differences across the protoplasm,<sup>4</sup> a great deal of information has been accumulated concerning their behavior under these conditions. It has been found that in a very great number of cases the capillary becomes plugged by a jelly-like material, probably derived from the protoplasm. This material seems to have little or no effect on the electrical properties of the system, but effectively prevents the escape of liquid from the vacuole. Because of this plug formation, the present experiments were extremely tedious and time consuming. By far the greater number of cells impaled plugged the capillary in a short time. In a few cases the plug was removed by cautious reaming with a fine tungsten wire, but in general the plug once formed was irremovable.

The cells were impaled on fine thin-walled capillaries drawn on the ends of thick-walled capillary tubes about 0.15 cm. in diameter and about 10 cm. long.

The cell impaled on the capillary was supported on a glass ring attached to the cork of a wide-mouth 4 oz. bottle, in such a way that the cell was immersed just below the surface of the sea water. A groove was cut in the side of the cork in order to keep the pressure in the bottle at that of the atmosphere. The bottles were immersed up to the neck in a large shallow tank, through which a moderately rapid flow of sea water was maintained. This served roughly as a thermostat keeping the temperature during the experiment at  $17.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ .

Before impalement the volume of each cell was determined separately by the method described in a previous paper.<sup>5</sup> At intervals, after impalement, measurements of the height of the liquid in the capillary were made, using a micrometer caliper reading to 0.002 cm. The liquid was allowed to rise only a few centimeters in the capillary before it was removed by means of a fine capillary tube. This prevented the development of too great a hydrostatic pressure.

The rise of liquid in the capillary was observed for about 48 hours in most cases, after which the cell sap was extracted for analysis for potassium and chloride. The former was determined on 0.1 ml. samples by micro-gravimetric analysis as  $\text{K}_2\text{PtCl}_6$  and the latter on 0.1 ml. samples by potentiometric titration with silver nitrate.

A group of cells from the same collection as the impaled cells was analyzed for potassium and halide with the following results,  $\text{K} = 0.5123 \text{ M}$ , halide =  $0.6166 \text{ M}$ . These figures were used in calculating the number of moles of potassium and halide initially present in each case. This procedure is not wholly satisfactory, since it neglects the natural variations in composition among the cells. However, since all cells used for impalement, for initial analysis, and for volume change of un-

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<sup>4</sup> Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, 11, 193.

<sup>5</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, 15, 537.

unpaled cells were derived from the same collection, and had been kept in running sea water in the laboratory under precisely similar conditions it is considered that the natural variations would not have been great enough to vitiate the calculations. This is certainly true of the halide calculations.

In this connection the recent analyses of Steward and Martin<sup>6</sup> are of interest. Their results show that the halide and potassium concentration of cells stored in the laboratory tended to become stabilized at fairly constant values.

TABLE I

*Increase in Volume and Moles of Potassium and Halide in Impaled Valonia Cells in 48 Hours*

Cell No	Increase in volume in 48 hrs.	Final molar concentration		Increase in (moles $\times 10^4$ )		Per cent increase in moles		Increase in volume
		Potassium	Halide	Potassium	Halide	Potassium	Halide	
	cc	M	M					per cent
1	0.198	0.4949	0.6171	0.844	1.226	21.1	25.5	25.5
2	0.124	0.4872	0.6162	0.461	0.763	15.8	21.7	21.7
3	0.062	0.5000	0.6183	0.270	0.388	16.1	19.2	18.9
4	0.133	0.4949	0.6109	0.586	0.789	27.5	30.8	32.0
5+6+7†	0.289	0.5156	0.6089	1.535	1.656	22.0	20.0	21.5
8+9+10†	0.471	0.5105	0.6175	2.372	2.925	25.9	26.6	26.4
Average						21.4	23.9	24.3

\* The increase in moles has been calculated as the difference between moles initially present and moles present at the end of the experiment. These data are omitted from the table but they can readily be calculated by calculating the initial and final volumes from the volume increase and per cent volume increase in moles  $\times 10^4 = \text{concentration} \times \text{volume in cubic centimeters} \times 10$ . Initial concentrations of K and halide were 0.5123 M and 0.6166 M respectively.

† The sap of these three cells was combined for analysis.

Another similar group of 10 cells was used to determine the volume change of unpaled cells kept under the same conditions.

In all the increase in volume of the sap of eighteen unpaled cells was followed. In some cases no analyses were made, as the sap was lost, and in others the sap was used for pH determination.

The experiments were carried out in Bermuda in the winter of 1936-37, in the

<sup>6</sup> Steward F. C., and Martin J. C. *Carnegie Institution of Washington, Pub No 475*, 1937, 117.

dim light of a basement laboratory The air temperature varied between 18° and 21°C but the bath in which the bottles were immersed was at 17.5°C  $\pm$  0.5°C

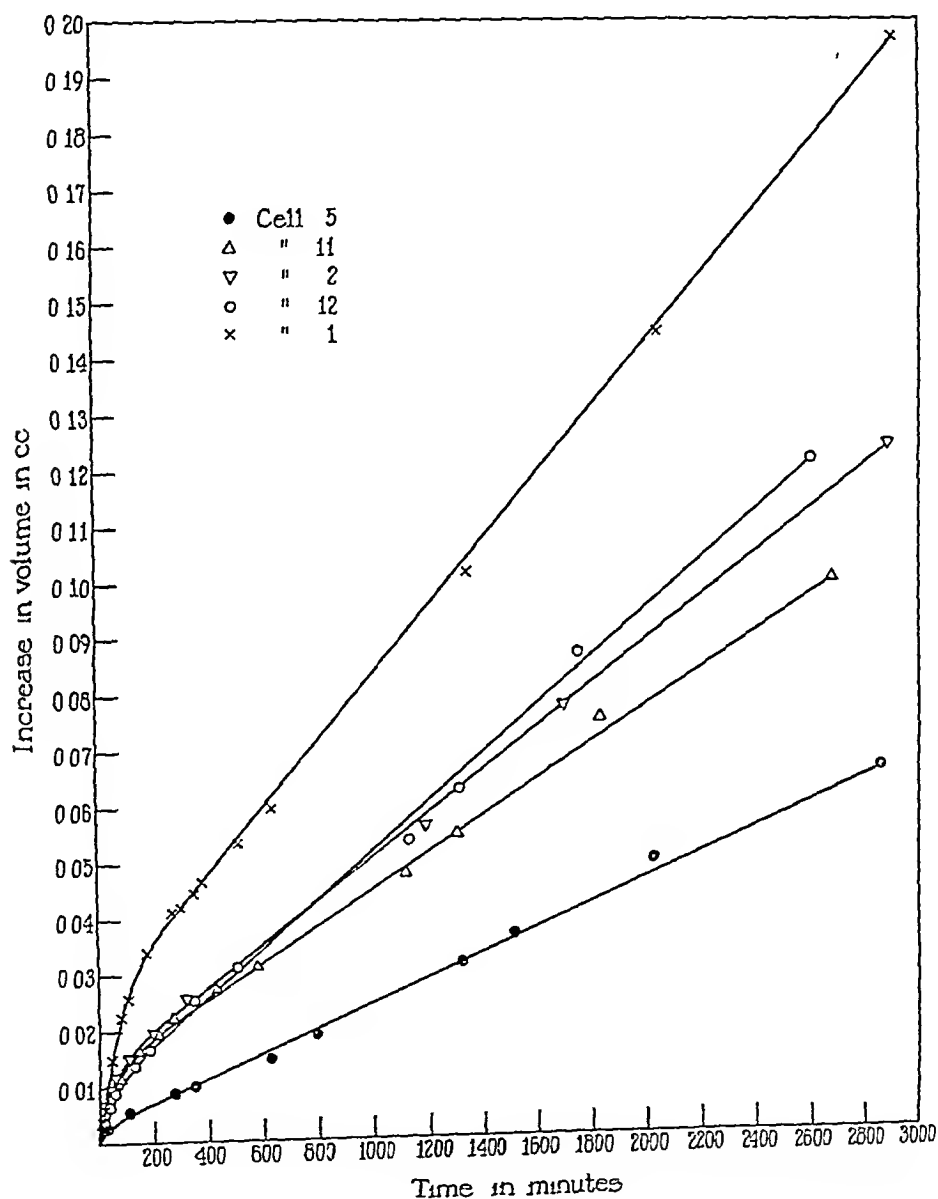


FIG 1 Rate of increase of sap volume of impaled cells of *Valonia macrophysa*

Some results are given in Fig 1 and Table I

In Table I we have included only the ten cells for which we have

sap analyses at the end of the exposure. And the total increase in the volume of the sap has been calculated from the rise of the liquid in the capillary by the usual formula

$$\text{Volume} = \pi r^2 h$$

where  $h$  is the capillary rise.  $r$  was measured directly by the microscope by means of a micrometer eyepiece. The number of moles of potassium and halide passing into each cell has been calculated as the difference between moles originally present in the sap and moles present at the end of the experiment.

Fig. 1 gives a selection of curves showing the increase in the volume of the sap with time. Fifteen cells were followed for the full 48 hour period. The average volume at the start was 0.491 cc. The largest cell volume was 0.779 cc and the smallest 0.209 cc. The average increase in volume was 0.121 cc or 24.7 per cent with an average deviation from the mean of 4.5 per cent. The smallest volume increase was 15.6 per cent and the largest 41.4 per cent. There was no discernible correlation between the size or shape of the cell and the rate of increase in volume.

The curves are typical. Curves not given have been omitted merely in order to avoid too much overlapping of points.

#### DISCUSSION OF RESULTS

Fig. 1 shows that the rate of increase of volume of the cell sap was greatest at the beginning. The initial rate, however, diminished in from 2 to 5 hours, and subsequently it was approximately linear. It seems probable that the first rapid increase of volume represents an adjustment of the osmotic pressure relationships of the sap and sea water due to dilution of the sap because water enters more rapidly than electrolyte. This would be expected when the restriction of the cellulose wall is removed.

A number of measurements of the freezing point depression of Bermuda sea water and of *Valonia* cells growing in it were made, while the experiments described in this paper were in progress. As anticipated from the fact that unimpaled cells have considerable turgidity it was found that the freezing point depression of the sap was always greater than that of the sea water.

The average freezing point of Bermuda sea water<sup>7</sup> is  $-2.021^{\circ}\text{C}$  which corresponds very closely to the freezing point of a  $0.603\text{ M}$ <sup>8</sup> KCl or NaCl solution. The average freezing point of the sap was  $-2.150^{\circ}\text{C}$  which corresponds to the freezing point of a  $0.640\text{ M}$  KCl or NaCl solution. In order to bring the concentration of the sap down to  $0.603\text{ M}$ , it would be necessary to dilute each  $1.000\text{ cc}$  to  $1.061\text{ cc}$ .

It seems highly probable that after the linear rate has been established, electrolyte and sap enter at such a rate as to keep the total concentration in the sap approximately constant.

The question now remains whether there is any evidence that the sap is diluted sufficiently during the early rapid entrance to bring the osmotic pressure of the sap down to that of the sea water.

Let us take the curve for cell 1. During the first 300 minutes (after which the rate became linear) the volume of the sap increased by  $0.042\text{ cc}$ . But the original volume was  $0.776\text{ cc}$ , so that if only water had entered the cell the concentration<sup>9</sup> would have dropped from  $0.640$  to  $0.607\text{ M}$ . But during this period electrolyte also entered and it may be assumed that the rate of entrance was not less than that during the linear part of the curve. In order to estimate the dilution we may break up the process of entrance in the rapid stage into two parts, (a) the dilution of the sap by pure water, and (b) the concurrent addition of undiluted sap to the sap. Interpolating from the linear part of the curve we find that in 300 minutes, the equivalent of  $0.018\text{ cc}$  of undiluted sap was added to the sap. The amount of pure water added was therefore  $0.042\text{ cc} - 0.018\text{ cc} = 0.024\text{ cc}$ . This would cause the concentration to fall to  $0.794 - 0.818$  of  $0.640 = 0.621\text{ M}$ . Similar calculations for the other curves yield similar results.

Apparently, therefore, the maximum possible dilution is not enough

<sup>7</sup> The freezing points were determined by means of a Heidenham thermometer calibrated at the Bureau of Standards.

<sup>8</sup> This figure has been derived by plotting the freezing point data given in Landolt, H., and Bornstein, R., *Physikalisch-chemische Tabellen*, Berlin, Julius Springer, 5th edition, (Roth, W. A., and Scheel, K.), 1923, vol. 2, 1446 and 1452, and interpolating from the linear curve obtained.

<sup>9</sup> This is the theoretical concentration in terms of KCl or NaCl derived from freezing point measurements.

to abolish the difference in osmotic pressure between the sap and sea water

Returning to the actual figures, the initial halide concentration was 0.6166 M. And the average halide concentration of the sap of impaled cells at the end of 48 hours was 0.6148 M<sup>10</sup>. This represents an even smaller dilution than that calculated. This may mean that during the linear stage, the electrolyte concentration gradually builds up after the first dilution. In view of the natural variation of the cells it is difficult to draw definite conclusions, but it seems possible that in spite of the impalement the osmotic pressure of the sap is maintained at a level higher than that of the sea water.

It was found that the unimpaled cells grew at the rate of 0.8 per cent per day during the first 48 hours. This rate was in fact maintained for 10 days, at the end of which time growth measurements were stopped. The halide content of the sap of these cells at the end was 0.6154 M.

By contrast the volume of the impaled cells grew at the average rate of 12 per cent per day or 15 times as fast. Moreover from Table I it appears that the percentage increase in the moles of halide was almost as great as the percentage increase in the sap volume, so that the entrance of electrolyte was also about 15 times as fast as into unimpaled cells.

It will be observed that the percentage increase in potassium moles was somewhat less than the volume increase. This may mean that the ratio of K/Na in the sap decreased during the experiment, on the other hand it may be the result of natural variation.

We may now consider the possible reasons for the striking difference in the rate of entrance of water and electrolytes into impaled and intact cells.

The method of treatment which follows is derived from the theoretical interpretations given by Osterhout<sup>11</sup> and by Longworth<sup>12</sup> for

<sup>10</sup> The discrepancy between the concentration 0.640 M which is the concentration of a solution of KCl or NaCl equivalent to that of the sap is made up in part by the nitrate concentration of the sap which is on the average 0.016 M. The remainder may be due to bicarbonate ion and other organic anions.

<sup>11</sup> Osterhout W. J. V. *J. Gen. Physiol.* 1932-33, 16, 529.

<sup>12</sup> Longworth L. G. *J. Gen. Physiol.* 1933-34, 17, 211.

the guaiacol model. The model consists of an "oily" layer (guaiacol) separating two aqueous layers. It seems probable that the *Valonia* cells and the surrounding sea water form essentially the same sort of system, with the important difference that in the unimpaled cell the vacuole is an enclosed space, not a free space as is the corresponding space in the model. From electrical considerations it seems probable that the protoplasm consists of "oily" surface layers separated by an aqueous layer. But for the present purpose it may be treated as a single "oily" layer. In the model it is assumed that at each phase boundary there are a pair of adjacent unstirred layers, one on the aqueous and the other on the non-aqueous side of the boundary. Similarly in the cell there are two pairs of adjacent unstirred layers, one pair at the sap-protoplasm interface and the other at the sea water-protoplasm interface. It is assumed that immediately at the interface between two unstirred layers the solutes are in equilibrium. In the equilibrium region the ratio of the concentration of a solute in the protoplasmic surface to its concentration in the external aqueous phase is its partition coefficient. It is assumed that ionization is negligible in the non-aqueous layers of the protoplasm so that solutes and water transferred between the sea water in the sap must diffuse in the non-aqueous portion of the protoplasm chiefly in the molecular form.

It is supposed that bases,  $M^+$ , are transferred between the sap and sea water in the form of  $MX$ , where  $X^-$  is the anion of a weak acid  $HX$  elaborated by the protoplasm.

For the flux of bases<sup>13</sup> we may derive the following equation

$$\frac{dM}{dt} = \frac{AD^{MX}k_{coll}}{h} \{S_{op}^{MX} [M]_o(OH)_o[HX]_{op} - S_{ip}^{MX} [M]_i(OH)_i[HX]_{ip}\} \quad (a)$$

and for the flux of water

$$\frac{dH_2O}{dt} = \frac{AD^{H_2O}}{h} \{S_{op}^{H_2O} [H_2O]_o - S_{ip}^{H_2O} [H_2O]_i\} \quad (b)$$

<sup>13</sup> Each base  $M^+$  is necessarily accompanied by anion, but this particular anion does not necessarily appear in the sap. In the model, for example, potassium moves through the guaiacol in the form of potassium guaiacolate (called for convenience KG), but it appears in the internal aqueous phase as potassium bicarbonate due to the decomposition of KG by carbonic acid. In the case of *Valonia* it is  $MCl$  which appears in the sap, but the method by which the  $Cl^-$  ion enters the sap is not important for the present discussion.

where  $D$  indicates the diffusion constant,  $S$  the partition coefficient,  $h$  the thickness of the diffusion layer, and  $A$  its area.  $o$  refers to the sea water,  $i$  to the sap,  $op$  to the aqueous unstirred layer at the sea water protoplasm interface, and  $ip$  to the aqueous unstirred layer at the sap protoplasm interface. Unless otherwise stated we are dealing in each case only with the part of the unstirred layer where the solutes are in equilibrium across the interface. Square brackets indicate concentrations and round brackets activities.

In equation (a)

$$*k_{\text{coll.}} = \frac{f_o^M f_{op}^{HX}}{f_{op}^{MX} k_{\text{hyd.}}(\text{H}_2\text{O})_{op}} = \frac{f_i^M f_{ip}^{HX}}{f_{ip}^{MX} k_{\text{hyd.}}(\text{H}_2\text{O})_{ip}}$$

The assumption is made that corresponding activity coefficients in the sap and sea water are equal since the ionic strengths of the two aqueous phases are sufficiently alike. The activity of water in the two phases is also assumed to be the same.

Equation (a) is derived in the following manner: we put

$$[MX]_p = S_{op}[MX]_{op}, \text{ and } [MX]_i = S_{ip}[MX]_{ip}$$

where  $p$  refers to the protoplasm side of the sea water protoplasm interface and  $i$  to the protoplasm side of the sap-protoplasm interface.

It is now assumed that

$$[M]_p = [M]_o, \text{ and } (\text{OH})_p = (\text{OH})_o \text{ and } [M]_i = [M] \text{ and } (\text{OH})_i = (\text{OH})_i$$

that is to say that for these ions derived from the sea water or sap there are no gradients in the aqueous unstirred layers. This will not be true of  $\text{H}^+$  or  $\text{X}^-$  ions which are derived from the protoplasm. And for these we must employ the concentrations in the aqueous layers in equilibrium, since these concentrations determine the rate of entrance.

Now  $\text{HX}$  is a weak acid. Consequently its salts are hydrolyzed in aqueous solution according to the general equation,



for which the formal thermodynamic mass action equation is

$$\frac{[M](\text{OH})[HX]_{op} f_o^M f_{op}^{HX}}{[MX]_{op}(\text{H}_2\text{O})_{op} f_{op}^{MX}} = \frac{[M]_i(\text{OH})[\text{HX}]_{ip} f_i^M f_{ip}^{HX}}{[MX]_{ip}(\text{H}_2\text{O})_{ip} f_{ip}^{MX}} = k_{\text{hyd.}}$$



Now

$$\begin{aligned}\frac{DM}{dt} &= \frac{AD^{MX}}{h} ([MX]_{po} - [MX]_{pi}) \\ &= \frac{AD^{MX}}{h} (S_{op} [MX]_{op} - S_{ip} [MX]_{ip})\end{aligned}$$

In this equation we substitute for  $[MX]_{op}$  and  $[MX]_{ip}$  the values obtained from the hydrolysis equation above and in this way obtain equation (a) for a cell with a protoplasmic surface of unit area and thickness

By making the reasonable assumptions that  $[HX]_{op} = [HX]_{ip} =$  constant, that  $S_{op}^{MX} = S_{ip}^{MX}$ , and that  $S_{op}^{H_2O} = S_{ip}^{H_2O}$  we can reduce equations (a) and (b) to

$$\frac{dM}{dt} = k_1([M]_o(OH)_o - [M]_i(OH)_i) \quad (c)$$

$$\frac{dH_2O}{dt} = k_2([H_2O]_o - [H_2O]_i) \quad (d)$$

The interdependence of these two processes will be clearer if we realize that equation (d) may be written

$$\frac{dH_2O}{dt} = k_2'(C_i - C_o) \quad (e)$$

in which  $C$  = the osmolar concentration Or

$$\frac{dH_2O}{dt} = k_2'(\mu^{MCl_i}[MCl]_i + \mu^m[m]_i - \mu^{MCl_o}[MCl]_o - \mu^m[m]_o) \quad (f)$$

where  $\mu$  is the osmotic coefficient,  $M = K + Na$ , and  $m$  = all osmotically active constituents except  $NaCl$  and  $KCl$ . Equations (c) and (f) may be compared with the equations developed by Jacobs<sup>14</sup> for the simultaneous penetration of a solute and water into cells<sup>15</sup>  $k_1$

<sup>14</sup> Jacobs, M. H., *J. Cell and Comp. Physiol.*, 1932-33, 2, 427, 1933-34, 4, 161

<sup>15</sup> The situation is complicated in the case of *Valonia* by the entrance of halide in some form. Tentatively we shall assume that halide passes through the protoplasm as  $NaCl$ , and that at the protoplasm-sap interface  $Cl$  is exchanged for  $Z$ , the anion of weak acid,  $HZ$ , which is found in the sap and that this process goes on at such a rate that all  $MOH$  entering the sap is transformed to  $MCl$ . Thus  $[MCl]_i \approx [M]_i$ .

and  $k'_2$  are not, as they are in Jacobs' equation, comparable "permeability constants" because the concentrations are not expressed in the same terms. However, the same considerations apply in certain respects.

Thus there are three main possibilities, (a)  $k_2$ , the permeability constant of water, may be much greater than  $k_1$ , the permeability constant of the electrolyte, (b)  $k_1$  may be very great compared with  $k_2$ , (c)  $k_2$  and  $k_1$  may be of the same order of magnitude.

In the case of *Valonia* the evidence suggests that condition (a) obtains since it is observed that in hypertonic sea water the cell softens very rapidly due to the loss of water, and that in hypotonic sea water it hardens very rapidly due to the gain of water.

Under these conditions, as Jacobs points out, the cell sap might be expected to be in osmotic equilibrium with the sea water since the entrance of electrolyte would be followed at once by the entrance of enough water to restore the balance. Approximately this condition is fulfilled in the impaled cell, except that apparently the osmotic pressure of the sap is slightly in excess of that of the sea water. This, however, is not definitely established. As pointed out earlier in the paper there is evidence that some osmotic adjustment takes place when the cell is impaled, but the concentration of the halide seems to decrease too little to produce osmotic equilibrium.

In both intact and impaled cells, the electrolyte may be considered to enter at a rate proportional to the gradient<sup>16</sup>

$$[M]_0(\text{OH})_0 - [M]_1(\text{OH})_1$$

But since the rate of electrolyte entrance is much less in the intact than in the impaled cell we should expect the gradient to be smaller in the intact cell. In this case therefore we should expect the concentration of  $MCl$ <sup>17</sup> in the sap to be much higher in the intact than in the impaled cell, since in this way the gradient would be smaller as required by the theory. This is contrary to observation since ap

<sup>16</sup> This hybrid expression containing activities and concentrations is used because the available data are the concentrations of potassium and sodium in the sap and sea water from analytical data and the hydrogen ion activities from pH measurements. The activity coefficients are not known.

proximately the same steady state is observed in both intact and impaled cells

An increase in the gradient could occur if the pH of the sap decreased on impalement. But within the limits of natural variation the pH of both impaled and intact cells seemed to be about the same. Since the carbon dioxide in the sap appears to be under slight pressure, impalement might be expected to relieve this pressure. This should, however, cause the sap pH to rise which is a change in the wrong direction.

Some figures may be illuminating. In an intact cell under normal conditions we may calculate the gradient for potassium entrance approximately from the following data,<sup>17</sup>

$$[K]_o = 0.012 \text{ M}, \quad (OH)_o = 10^{-6}, \quad [K]_i = 0.5 \text{ M}, \quad (OH)_i = 10^{-8}$$

Hence the gradient

$$[K]_o(OH)_o - [K]_i(OH)_i = (12 \times 10^{-9}) - (5 \times 10^{-9}) = 7 \times 10^{-9}$$

Obviously no change in  $[K]_i$  or  $(OH)_i$  can bring about a 15-fold increase in the gradient since even if the right hand term vanishes the increase will be less than 2-fold.

In the impaled cell the gradient has approximately this value and one-fifteenth of it is  $4.7 \times 10^{-10}$ . Hence to attain this value the internal product would have to increase to  $11.53 \times 10^{-9}$ . If the sap pH remains at 6.0 this can occur if  $[K]_i$  increases from 0.5 M to 1.15 M, or if  $[K]_i$  remains constant at 0.5 M by an  $(OH)_i$  increase to  $23 \times 10^{-9}$  corresponding to a pH of 6.36. This is conceivable but not likely since because of the pressure developed in the intact cell its pH is likely to be less than that of the impaled cell. Actually the pH of the sea water especially in the outer protoplasmic layer is apt to be somewhat greater than that of the main mass of the sea water, during illumination, since carbon dioxide may be withdrawn from that layer photosynthetically. If for example immediately at the sea water-protoplasm interface, the pH is 9.0 (which it may well be since we have evidence that by photosynthesis the pH of even a large amount of sea water in a stoppered<sup>18</sup> bottle may be raised as high as 9.5) the gradient would be  $(120 \times 10^{-9}) - (5 \times 10^{-9}) = 115 \times 10^{-9}$ . One-fifteenth of this is  $7.7 \times 10^{-9}$ . To obtain this value the right hand term of the gradient equation must be  $112.3 \times 10^{-9}$ , so that if the pH remains constant  $[K]_i$  must increase to 11.23 M or if  $[K]_i$ ,

<sup>17</sup> Calculations for entrance of sodium which is going on simultaneously are omitted since they add nothing new.

<sup>18</sup> See also Crozier, W. J., *J. Gen. Physiol.*, 1918-19, 1, 581.

remains constant the pH must increase to 7.35. Obviously these are impossibly large changes.

The third case (c) where  $k_1$  (the rate constant for the electrolyte) and  $k_2$  (the rate constant for the water) are of the same order of magnitude can be dealt with briefly since it resembles case (a) closely. In the impaled cell where water can enter freely, the concentration in the sap is determined by the relative magnitudes of  $k_1$  and  $k_2$ , as it is in the guaiacol model. We should not expect approximate osmotic equality at this steady state except as the result of pure chance.

It is therefore interesting that in the case of *Halicystis*, also, to be discussed elsewhere, the sap and sea water in the impaled cell are approximately in osmotic equilibrium. As in case (a) where  $k_1$  is very small compared with  $k_2$ , when the cell is intact we should expect the gradient to be smaller in agreement with the slower rate of electrolyte entrance. This, as pointed out before, would necessitate an increase either in the  $MCl$  concentration or the pH of the sap. Neither of these things is observed.

In case (b)  $k_1$  is extremely large compared with  $k_2$ . On this basis we should expect the gradient  $[M](OH) - [M](OH)$  to be very nearly zero at all times since the entrance of water would be followed at once by the entrance of enough electrolyte to abolish the gradient.

Considering potassium only and using the data given above the minimum concentration of potassium in the sap which would satisfy the condition of zero gradient would be 1.2 M. In this case, since the gradient is already maintained close to zero, it would require only a very small change in  $MCl$  or in the pH of the sap to decrease it to one fifteenth of its value in the impaled cell. Such a change might easily go undetected analytically. Thus theoretically we could have in this case the same steady state in both intact and impaled cells within the limits of experimental error. But the concentration level of the sap would be far above that of the sea water. This has never been observed.

The foregoing leads to the conclusion that it is impossible to explain the increased rate of electrolyte and water entrance in the impaled cell from a consideration of gradients alone.

In seeking for other explanations we have considered the possibility that impalement produces changes in the protoplasm which lead to changes in its permeability

We may assume that in the impaled cell water enters so rapidly that any increase in osmotic pressure of the sap just inside the protoplasm is at once abolished by the entrance of water. In the intact cell where the entrance of water is limited by the slow growth of the cell electrolyte tends to raise the osmotic pressure of the sap much more. The cellulose envelope can be stretched up to a certain point and a little water can be added to increase the volume of the sap in this way. But the limit to which the envelope can be stretched is soon reached. Thereafter as more electrolyte enters the vacuole tends to gain water at the expense of the protoplasm. For simplicity we may assume that when the sap gains in volume by taking water from the protoplasm, the protoplasm decreases in volume by the same amount.<sup>19</sup> We assume also that because of the high rate of diffusion of water in the protoplasm, any water gradient set up by the withdrawal of water into the sap is at once abolished. Thus the state of hydration is uniform throughout the non-aqueous part of the protoplasm.

The protoplasm has three layers with an aqueous layer  $W$  enclosed between two non-aqueous layers,  $X$  at the sea water side and  $Y$  at the sap side. At first sight it might seem that water would be withdrawn from  $W$  rather than from  $X$  and  $Y$ . But it must be remembered that  $W$  is not pure water but is a solution, and it is fair to assume that it is in approximate equilibrium with both the sea water and the sap. Any water it may lose will therefore be replaced from these phases, and as far as  $W$  is concerned  $X$  and  $Y$  serve merely as osmotic membranes separating it from the sea water and sap.

We may picture the dehydration process as follows. The cell wall has already been stretched to its greatest extent but a little more electrolyte enters the sap and raises its osmotic pressure. As a result some water is removed from the  $Y$  layer, in turn some water moves from the  $W$  layer to  $Y$ , and is at once replaced by water moving into

<sup>19</sup> Not quite true since the components do not mix in all proportions and therefore do not form an ideal solution. The effect of the slight volume changes which occur need not be taken into consideration here.

$W$  from  $\lambda$ . But  $X$  cannot regain the water it loses because the cell wall is already stretched, and if water enters  $X$  it will cause an increase in the volume of the sap plus the protoplasm, which is impossible until the cell wall grows. These processes presumably take place so rapidly that the dehydration takes place uniformly throughout the non aqueous protoplasm, and it should be clear that  $W$  contributes little or no water to the sap.

The dehydration of the protoplasm may be supposed to have two important effects

(a) The viscosity of the non aqueous layers of the protoplasm may be increased and thereby the diffusion constants of each diffusing molecular species may be decreased

(b) The solubility of each diffusing species in the non aqueous protoplasmic layers may be decreased thereby decreasing the partition coefficient of each species. This effect would decrease the gradient of concentration of a species in the protoplasm.

Reduced to its essentials the rate equation for electrolyte entrance may be written for a cell surface of unit area and thickness,

$$\frac{dM}{dt} = D^{MX} S^{MX} ([MX]_o - [MX]_i)$$

This may be written

$$\frac{dM}{dt} = k_{\text{eff}} ([M]_o(\text{OH})_o - [M]_i(\text{OH})_i)$$

of which the gradient term remains reasonably constant. Therefore if either  $D^{MX}$  or  $S^{MX}$  decreases the rate of electrolyte entrance decreases.

In the impaled cell we may suppose that the non aqueous protoplasm is saturated with water at all times, but in the intact cell the degree of hydration is changing continually. And in the limit we assume that it may be so low that the protoplasm is at times impermeable to some at least of the electrolytes which normally enter.

The changes in rate in response to changes in the degree of hydration of the non aqueous protoplasm may allow the cell to maintain the osmotic concentration of the sap at a nearly constant level whether the cell is impaled or intact. The composition of the cell

sap, however, is the resultant of the diffusion of a number of molecular species at various rates, to and from the vacuole. We should scarcely expect that the dehydration of the protoplasm would affect the diffusion and partition coefficient of each species relatively to the same extent, so that when a cell is impaled and takes in electrolytes its sap composition might be expected to change. We did not observe such a change, but 48 hours may have been too short a period for an observable alteration, especially as the sap composition of normal intact cells is variable,<sup>20</sup> although the osmotic concentration is not. It would be a formidable undertaking to carry out experiments for longer periods, because of the great difficulty in keeping the capillary from plugging.

There is also the possibility that the permeability of the protoplasm remains unchanged to all diffusing species until a critical degree of hydration is reached, whereupon it becomes temporarily impermeable to all species. This would account for the constant composition.

It is also possible that there are other consequences of impalement which affect the permeability of the protoplasm. Thus it has been suggested to us by L. G. Longworth<sup>21</sup> that since the turgor pressure disappears on impalement the state of compressibility of the protoplasm is changed.

#### SUMMARY

When *Valonia* cells are impaled on capillaries, it is in some ways equivalent to removing the comparatively inelastic cellulose wall. Under these conditions sap can migrate into a free space and it is found that on the average the rate of increase of volume of the sap is 15 times what it is in intact cells kept under comparable conditions.

The rate of increase of volume is a little faster during the first few hours of the experiment, but it soon becomes approximately linear and remains so as long as the experiment is continued.

The slightly faster rate at first may mean that the osmotic pressure of the sap is approaching that of the sea water (in the intact cell the sap osmotic pressure is always slightly above that of the sea water). This might result from a more rapid entrance of water than of

<sup>20</sup> See Stewart, F. C., and Martin, J. C., *Carnegie Institution of Washington, Pub. No. 475*, 1937, 87.

<sup>21</sup> Private communication.

electrolyte, as would be expected when the restriction of the cellulose wall was removed

During the linear part of the curve the osmotic concentration and the composition of the sap suffer no change, so that entrance of electrolyte must be 15 times as fast in the impaled cells as it is in the intact cells

The explanation which best accords with the facts is that in the intact cell the entrance of electrolyte tends to increase the osmotic pressure. As a consequence the protoplasm is partially dehydrated temporarily and it cannot take up more water until the cellulose wall grows so that it can enclose more volume. The dehydration of the protoplasm may have the effect of making the non aqueous protoplasm less permeable to electrolytes by reducing the diffusion and partition coefficients on which the rate of entrance depends. In this way the cell is protected against great fluctuations in the osmotic concentration of the sap





# THE PARTIAL REACTIVATION OF FORMOLIZED TOBACCO MOSAIC VIRUS PROTEIN

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Considerable evidence has accumulated indicating that virus activity is a specific property of the tobacco mosaic virus protein (1), yet little information is available on the relationship between protein structure and virus activity. Certain reagents inactivate the virus, and at least part of the changes they bring about in the protein molecule have been determined (2). In no case, however, has the nature of the specific change that causes loss of activity been established. The reactivation of inactivated virus would appear to be one of the simplest means of obtaining direct evidence relating virus activity to protein structure. Should it prove possible to alter the protein by some chemical means that also destroys virus activity, to demonstrate that the protein is altered and to establish the chemical nature of the change, and then to restore the protein molecule to its original form with an accompanying restoration of virus activity, it would serve to correlate directly virus activity with the structure of the protein.

It has been shown that tobacco mosaic virus protein inactivated by hydrogen peroxide, ultraviolet light, formaldehyde, or nitrous acid is only slightly altered and retains certain chemical and serological properties characteristic of the active virus protein (2). The inactive proteins can be crystallized, are soluble, are still native, and give positive precipitin reactions with the sera of animals injected with active virus preparations. Dr Lauffer<sup>1</sup> has found that the stream double refraction of formolized virus protein is the same or slightly less than that of active virus protein, and it has been shown that the sedimentation constant of tobacco mosaic virus protein inactivated

<sup>1</sup> Personal communication (see also *J Biol Chem*, 1938, 123, 507)

by hydrogen peroxide, formaldehyde, or nitrous acid (3) is practically the same as that of the active protein. These reagents must then cause only minor changes in the protein molecule, hence it seemed possible that the reactions caused by some of them might be reversible. The reaction of formaldehyde with proteins or with amino acids has been shown to be partially reversible by Holden and Freeman (4) and by Wadsworth and Pangborn (5). Anderson (6) has shown that, while the condensation between formaldehyde and gelatin is fairly stable in alkaline solutions, it is less stable in acid solutions. Schultz and Gebhardt (7) stated in a preliminary note that they had obtained reactivation of formolized bacteriophage merely by dilution. The reaction between the virus protein and formaldehyde was, therefore, selected as the one most likely to be reversible as well as one of a type that might permit the determination of the chemical changes that occur. The present paper records the results obtained in a study of the inactivation of tobacco mosaic virus protein with formaldehyde and the reactivation of the formolized protein.

#### EXPERIMENTAL

*Preparation of Virus Protein*—The tobacco mosaic virus protein used in the present experiments was isolated from diseased Turkish tobacco plants either by a chemical method (8) or by means of an ultracentrifuge (9). In the first method the virus was precipitated from the filtered dilute dipotassium phosphate extract of the frozen macerated plants with ammonium sulfate and the precipitate was dissolved in M/10 phosphate buffer at pH 7. The precipitation was repeated until the filtrate remained colorless, after which the virus protein was adsorbed on celite at pH 4.5 and then eluted at pH 7. By working in a cold room and keeping all preparations below 5°C, colorless preparations were obtained without the use of calcium oxide. In the second method the protein was isolated from the filtered extract by alternate high and low speed centrifugation. Preparations were dialyzed at least 24 hours against flowing distilled water before they were used and, as they contained no detectable amounts of non-protein nitrogen, protein estimations were calculated from total nitrogen determinations.

*Determination of Activity*—The half-leaf method of inoculation (10, 11) as used in this laboratory (12) was employed, with the exception that whole leaves were inoculated when testing for complete or nearly complete inactivation. *Nicotiana glutinosa* L. was selected for use as the test plant in most of the experiments because it was found to be affected less by the toxic action of high concentrations of inactive virus protein than was *Phaseolus vulgaris* L., var. Early Golden Cluster. Since it proved necessary to determine to what extent the production of toxic

protein accounts for the loss of activity, the effect of known amounts of inactive protein on the number of lesions obtained with virus protein was studied. Mixtures were made of active virus protein with various amounts of formaldehyde-inactivated virus protein, hydrogen peroxide inactivated virus protein, or egg albumin and the virus activity of the mixtures compared with that of active virus protein on both the above mentioned test plants by means of the half leaf method. It may be seen from the results presented in Table I that a concentration of  $10^{-3}$  gm of inactive protein per cubic centimeter had little if any effect on activity measurements when *Nicotiana glutinosa* was used, but that the effect was appreciable

TABLE I

*Effect of Different Concentrations of Various Inactive Proteins on the Activity of Tobacco Mosaic Virus Protein*

Inactive protein used	Concentration (gm protein/cc.) of mixtures		<i>Nicotiana glutinosa</i>				<i>Phaseolus vulgaris</i>			
			No of half leaves	Lesions		A/B $\times 100^\dagger$	No of half leaves	Lesions		C/D $\times 100^\dagger$
	Active	Inactive		A Mixture	B Control			C Mixture	D Control	
Formolized virus protein	$10^{-6}$	$10^{-3}$	27	371	737	50.5	33	337	1049	31.8
	$10^{-4}$	$10^{-3}$	31	917	1090	83.8	27	637	876	72.7
	$10^{-6}$	$10^{-4}$	33	579	929	62.3				
	$10^{-5}$	$10^{-3}$	30	529	501	105.6	35	529	765	69.1
	$10^{-4}$	$10^{-4}$	34	900	892	100.9	24	342	402	82.5
$H_2O_2$ -inactivated virus protein	$10^{-6}$	$10^{-3}$	29	207	543	38.1	32	79	566	13.9
	$10^{-6}$	$10^{-5}$					22	211	577	36.5
	$10^{-4}$	$10^{-3}$	24	153	597	25.6	38	460	3345	13.8
Egg albumin	$10^{-6}$	$10^{-3}$	27	807	1194	66.7	32	248	1018	24.5
	$10^{-6}$	$10^{-3}$	27	894	1352	66.3	32	558	1805	30.8
	$10^{-6}$	$10^{-3}$	31	547	893	61.3	41	2655	7392	35.9

\* All controls contained  $10^{-5}$  gm active protein per cubic centimeter

† The figures in this column are regarded as a measure of the relative activities of the mixtures when tested on the host indicated

with *Phaseolus vulgaris*. The formolized virus protein caused a smaller reduction in activity than did hydrogen peroxide inactivated virus protein and had about the same or a slightly greater effect than egg albumin. Regardless of whether the action of the inactive protein was an effect on the plant (13-14), an effect on the virus or a mechanical effect, it is evident that this production of a mildly toxic protein cannot account for the failure of formolized tobacco mosaic virus protein to exhibit virus activity and also that the effect of inactive protein on virus activity can be measured and a correction factor determined.

In the experiments to be reported the activity of a given preparation was

determined by inoculating against a control containing  $10^{-5}$  or  $10^{-6}$  gm of active virus protein per cubic centimeter and the results interpreted on the assumption that over the range used the number of lesions is proportional to the virus concentration. That such a proportionality exists over the range of  $10^{-5}$  to  $10^{-6}$  gm/cc has been shown by several investigators (15, 16). It was necessary to introduce a correction factor when preparations containing large amounts of inactive protein were used. This factor was obtained from data such as those presented in Table I and in Fig 1. Curve A of Fig 1, a dilution curve of one of the preparations used,

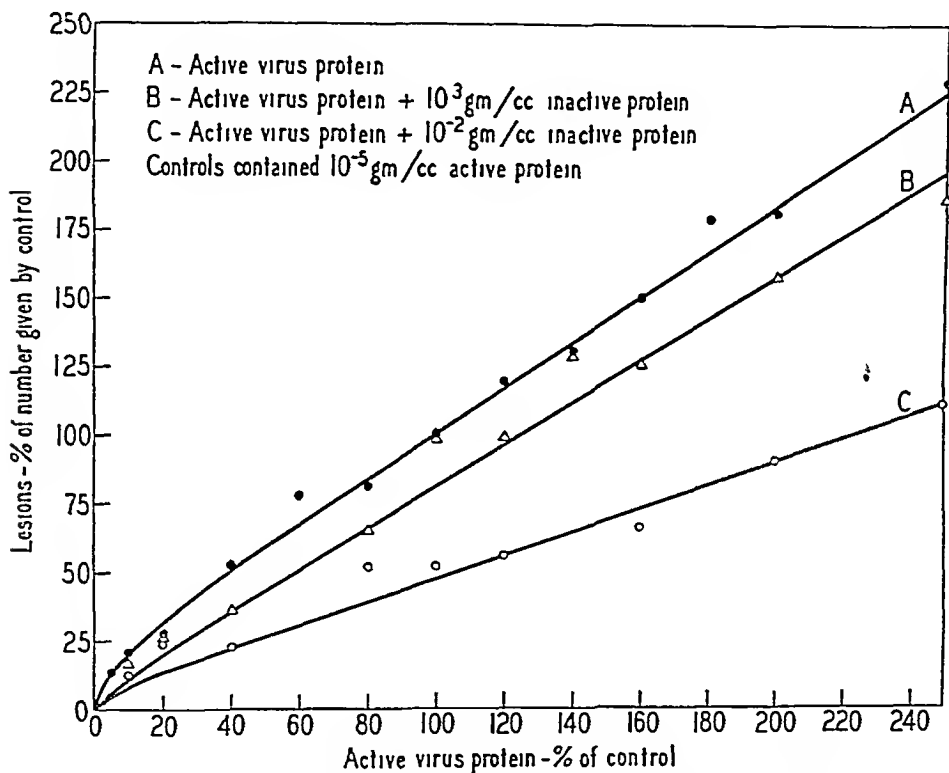


FIG 1 The effect of formaldehyde-inactivated virus protein on the activity of tobacco mosaic virus protein

was obtained by comparing the number of lesions given by different amounts of active virus protein with the number given by a control containing  $10^{-5}$  gm of active protein per cubic centimeter when inoculated to *Nicotiana glutinosa* using the half-leaf method. The number of lesions obtained in each case is expressed as per cent of those caused by the control. The figure shows that such a curve is not far from a straight line of slope 1 and that the error introduced in assuming that such is the case is not great. The other two curves were obtained in the same manner, except that definite amounts of completely inactive formolized virus

protein were added to each inoculum (but not to the controls) As may be seen from Fig 1, a concentration of  $10^{-3}$  gm of inactive protein per cubic centimeter had but slight effect on the dilution curve of active virus In many preparations this amount had no measurable effect on the virus activity A concentration of  $10^{-2}$  gm of inactive protein per cubic centimeter caused a constant percentage reduction of about 50 per cent in the number of lesions that was obtained Other preparations gave similar curves Therefore, with preparations containing  $10^{-3}$  gm of inactive protein per cubic centimeter no correction was made, while with those containing  $10^{-1}$  gm/cc. the figures for apparent activity were multiplied by a factor of 2 In most experiments involving the comparison of different samples, an attempt was made to dilute the samples so that approximately the same portions of the curves were used

*Inactivation*—Unless otherwise stated, inactivation was allowed to proceed at room temperature in mixtures of approximately 2 per cent protein and 2 per cent formaldehyde in  $M/10$  phosphate buffer at pH 7 Immediately after mixing the reactants a sample of the mixture was removed, diluted to  $10^{-5}$  or  $10^{-6}$  gm of protein per cubic centimeter, and inoculated against a control of the same concentration At intervals samples were removed for immediate dilution and inoculation and for dialysis against flowing cold distilled water for 6 hours in a Kunitz-Simms rocking type apparatus (17) to stop the reaction by removal of free formaldehyde After dialysis the samples were analyzed for nitrogen and diluted to suitable concentrations for inoculation against controls consisting of  $10^{-5}$  or  $10^{-6}$  gm of the original active virus protein per cubic centimeter Data on rate of inactivation are given in Tables II and III The latter table presents the data obtained in an experiment in which duplicate samples were used and is included as an illustration of the uniformity of results that may be expected under the conditions of the experiment It may be seen that in view of the accuracy of activity determinations (12) and of other factors the results agree quite well During the first 12 to 18 hours in which over 99 per cent of the protein is inactivated, the rate of inactivation follows roughly that of a monomolecular reaction However, a much greater length of time is required for complete inactivation than would be predicted on such a basis For this and other reasons it is not implied that the reaction is monomolecular, and the expression is used merely to describe the general shape of the inactivation curve There are not sufficient data at hand to permit a determination of the exact type of the reaction

Although it was found that dialysis removed the excess formaldehyde quite rapidly from the reaction mixture, it appeared desirable to determine whether or not the dialyzed preparations that were used for inoculation contained sufficient quantities of free formaldehyde to affect their activities Tests on the dialysates showed that after  $5\frac{1}{2}$  hours of dialysis against cold distilled water, the solutions con

TABLE II

*Rate of Inactivation of Tobacco Mosaic Virus Protein (1.86 Per Cent) by 2 Per Cent Formaldehyde at pH 7.0 and at Room Temperature*

Experiment	Time	No. of half-leaves	Concentration at which inoculated (gm protein/cc)		Lesions			Activity remaining
			Sample	Control	A Sample	B Control	A/B $\times 100^*$	
4†	hrs							per cent
	0†	27	$10^{-6}$	$10^{-6}$	326	328	100	100
	6†	28	$10^{-5}$	$10^{-6}$	599	497	120.7	12
	12†	14	$10^{-4}$	$10^{-6}$	419	385	108.8	1
	24†	32	$10^{-3}$	$10^{-6}$	108	248	43.6	0.04
	48†	40	$10^{-2}$		22			
	48	38	$10^{-2}$		60			
	72	42	$10^{-2}$		12			
	96	40	$10^{-2}$		3			
	192	38	$10^{-2}$		0			0.0
	0†	13	$10^{-5}$	$10^{-5}$	183	211	87	87
	6†	14	$10^{-4}$	$10^{-5}$	127	218	58	6
	6	25	$2 \times 10^{-4}$	$10^{-5}$	406	477	85	4
	12†	28	$10^{-4}$	$10^{-5}$	47	58	81	0.8
5	12	27	$2 \times 10^{-4}$	$10^{-5}$	628	541	116	0.6
	24†	30	$10^{-3}$	$10^{-5}$	34	112	30	0.03
	24	28	$5 \times 10^{-3}$	$10^{-5}$	110	267	41	0.01
	192	26	$10^{-2}$		0			0.0
	5	30	$10^{-4}$	$10^{-5}$	1704	741	230.0	23
	10	28	$10^{-3}$	$10^{-5}$	192	548	35.0	0.35
12‡	24	30	$10^{-2}$	$10^{-5}$	153	948	16.1	0.03
	96	30	$10^{-2}$		6			
	120	34	$10^{-2}$		2			
	144	30	$10^{-2}$		0			0.0
	168	26	$10^{-2}$		0			0.0
	5	33	$10^{-5}$	$5 \times 10^{-6}$	153	499	31.3	15
20‡	8	32	$10^{-4}$	$5 \times 10^{-6}$	340	479	71.0	3.5
	12	34	$10^{-3}$	$5 \times 10^{-6}$	221	489	45.4	0.23
	24	34	$10^{-2}$	$5 \times 10^{-6}$	30	463	6.5	0.006
	48	34	$10^{-2}$	$10^{-6}$	4	230	1.7	0.0003

\* The figures in this column represent the relative activities of the samples when compared with the controls at the concentrations indicated with *Nicotiana glutinosa* as test plant

† HCHO not removed from samples used for inoculation

‡ Ultracentrifugally prepared virus protein used in these experiments

tained less than  $10^{-5}$  gm of free formaldehyde per cubic centimeter. That this amount was too small to affect the activity of the preparations is evident from the data presented in Tables II and III, for many of the samples were used for inoculation without removal of the excess formaldehyde. Samples removed immediately after mix

TABLE III

*Rate of Inactivation of Tobacco Mosaic Virus Protein (1.86 Per Cent) by 2 Per Cent Formaldehyde at pH 7.0 and at Room Temperature*

Hours	Sample	Concentration (gm protein/cc.)		No of half leaves	Lesions			Activity remaining
		Sample	Control		C Sample	D Control	C/D $\times$ 100†	
0	A†	$10^{-4}$	$10^{-4}$	30	1063	919	116.8	117
	B†	$10^{-5}$	$10^{-5}$	28	1106	1108	99.8	100
6	A†	$10^{-4}$	$10^{-4}$	29	684	1328	51.5	5
	B†	$10^{-4}$	$10^{-4}$	28	821	1136	72.2	7
	A	$10^{-4}$	$10^{-5}$	29	776	1170	66.3	7
	B	$10^{-4}$	$10^{-5}$	29	760	983	77.3	8
12	A†	$10^{-3}$	$10^{-5}$	28	643	1080	59.3	0.6
	B†	$10^{-3}$	$10^{-5}$	29	529	1124	47.1	0.5
	A	$10^{-3}$	$10^{-5}$	26	474	1162	40.8	0.4
	B	$10^{-3}$	$10^{-5}$	29	741	1251	59.2	0.6
24	A	$10^{-3}$	$10^{-5}$	27	360	669	53.8	0.11
	B	$10^{-3}$	$10^{-5}$	26	284	698	40.7	0.08

\* Samples A and B were prepared from the same virus preparation and were adjusted to the same protein, formaldehyde phosphate, and hydrogen ion concentrations.

† The figures in this column represent the relative activities of the samples when compared with the controls at the concentrations indicated with *Nicotiana glutinosa* as test plant.

‡ Excess formaldehyde not removed from samples used for inoculation.

ing and containing  $10^{-5}$  gm free formaldehyde per cubic centimeter when inoculated were fully as infectious as the corresponding controls containing no formaldehyde. Further inspection of the tables reveals that solutions containing higher concentrations of free formaldehyde were practically as active as corresponding samples from which form



TABLE II

*Rate of Inactivation of Tobacco Mosaic Virus Protein (1.86 Per Cent) by 2 Per Cent Formaldehyde at pH 7.0 and at Room Temperature*

Experiment	Time	No. of half-leaves	Concentration at which inoculated (gm protein/cc)		Lesions			Activity remaining
			Sample	Control	A Sample	B Control	A/B $\times 100^*$	
	<i>hrs</i>							<i>per cent</i>
4†	0†	27	$10^{-6}$	$10^{-6}$	326	328	100	100
	6†	28	$10^{-5}$	$10^{-6}$	599	497	120.7	12
	12†	14	$10^{-4}$	$10^{-6}$	419	385	108.8	1
	24†	32	$10^{-3}$	$10^{-6}$	108	248	43.6	0.04
	48†	40	$10^{-2}$		22			
	48	38	$10^{-2}$		60			
	72	42	$10^{-2}$		12			
	96	40	$10^{-2}$		3			
	192	38	$10^{-2}$		0			0.0
5	0†	13	$10^{-5}$	$10^{-5}$	183	211	87	87
	6†	14	$10^{-4}$	$10^{-5}$	127	218	58	6
	6	25	$2 \times 10^{-4}$	$10^{-5}$	406	477	85	4
	12†	28	$10^{-4}$	$10^{-6}$	47	58	81	0.8
	12	27	$2 \times 10^{-4}$	$10^{-6}$	628	541	116	0.6
	24†	30	$10^{-3}$	$10^{-6}$	34	112	30	0.03
	24	28	$5 \times 10^{-3}$	$10^{-5}$	110	267	41	0.01
	192	26	$10^{-2}$		0			0.0
12‡	5	30	$10^{-4}$	$10^{-5}$	1704	741	230.0	23
	10	28	$10^{-3}$	$10^{-5}$	192	548	35.0	0.35
	24	30	$10^{-2}$	$10^{-5}$	153	948	16.1	0.03
	96	30	$10^{-2}$		6			
	120	34	$10^{-2}$		2			
	144	30	$10^{-2}$		0			0.0
	168	26	$10^{-2}$		0			0.0
20‡	5	33	$10^{-5}$	$5 \times 10^{-6}$	153	499	31.3	15
	8	32	$10^{-4}$	$5 \times 10^{-6}$	340	479	71.0	3.5
	12	34	$10^{-3}$	$5 \times 10^{-6}$	221	489	45.4	0.23
	24	34	$10^{-2}$	$5 \times 10^{-6}$	30	463	6.5	0.006
	48	34	$10^{-2}$	$10^{-6}$	4	230	1.7	0.0003

\* The figures in this column represent the relative activities of the samples when compared with the controls at the concentrations indicated with *Nicotiana glutinosa* as test plant

† HCHO not removed from samples used for inoculation

‡ Ultracentrifugally prepared virus protein used in these experiments

virus preparations were subjected to the above treatment, their activity was either unchanged or decreased less than 20 per cent

It became apparent early in the investigation that the amount of reactivation obtainable was dependent to some degree upon the extent to which inactivation had proceeded. In Table IV are included the data on reactivation of preparations retaining approximately 10 per cent of their original activity. In no case was the activity restored in full, but some reactivation was obtained in all cases, although in certain instances the differences may not be significant. The values given for the activity of the preparations are numbers calculated from the data and are regarded as a measure of the order of magnitude of the true activities. In most cases the differences are too large and obtained too often to be accounted for by errors of the method. It should be noted that Tables IV-VII record representative data on attempts at reactivation and do not give only the most favorable data. The numbers in the last column of each of Tables IV-VI are the ratios of the activities of the reactivated samples to those of the corresponding samples before reactivation. Each represents the number of times the activity of the designated sample was increased by the reactivation process.

Similar data on preparations possessing about 1 per cent of their original activity are presented in Table V. It may be seen that the results are more consistent and the differences obtained considerably larger. A 10 fold increase in activity was obtained in most cases, although the actual amount of reactivation in terms of active protein "created" is less than was obtained with some of the more active preparations. Due to the fact that it is possible to inoculate at different levels of protein concentration, it is as easy to distinguish between 1 per cent and 2 per cent active preparations as it is to distinguish between 50 per cent and 100 per cent active preparations. An increase from about 1 per cent activity to 10 per cent activity is quite significant and indicates a marked increase in the actual activity of the virus preparation.

As may be seen from the results that are presented in Table VI, essentially the same degree of reactivation was obtained with virus protein preparations possessing about 0.1 per cent of the original activity. In these experiments it was necessary to introduce a cor-







rection factor because of the effect of inactive protein on the lesion count. While this may reduce the accuracy of the values obtained for the activities of such preparations, there can be little doubt that an increase in activity followed reactivation. The differences can also be demonstrated by inoculating one side of the leaves of plants with a preparation before reactivation and the other side with the corresponding preparation after reactivation without further treatment or dilution.

TABLE VII

*Reactivation of Inactive or Nearly Inactive Formulated Tobacco Mosaic Virus Protein Preparations*

Experiment	Before reactivation			After reactivation		
	No of leaves	Concentration (gm protein/cc)	Lesion per leaf	No of leaves	Concentration (gm protein/cc)	Lesions per leaf
5	15	$10^{-1}$	0.0	15	$10^{-1}$	0.0
7b	12	$0.7 \times 10^{-2}$	0.2	15	$0.7 \times 10^{-2}$	57.5
7b	15	$0.7 \times 10^{-2}$	0.0	14	$0.7 \times 10^{-2}$	16.1
12a	15	$10^{-2}$	0.4	14	$10^{-2}$	8.0
12a	14	$10^{-2}$	0.0	14	$10^{-2}$	1.7
12b	15	$10^{-2}$	0.0	15	$10^{-2}$	2.1
12b				14	$10^{-2}$	0.21
12c	15	$10^{-2}$	0.0	15	$10^{-2}$	0.47
12c				15	$10^{-2}$	0.14
20†	13	$0.5 \times 10^{-2}$	0.4	15	$0.5 \times 10^{-2}$	4.0

\* *Nicotiana glauca* used as test plant

† Ultracentrifugally prepared virus protein used in this experiment

The activities of preparations less active than those already considered are difficult to measure accurately, but reactivation of such preparations can be demonstrated by inoculating whole leaves with the preparations before and after reactivation. The results obtained are given in Table VII. It was not found practicable to use higher concentrations than  $10^{-1}$  gm protein per cubic centimeter. Samples that retained some activity were invariably reactivated to an appreciable extent, but, if the inactivation had proceeded too far, reactivation was not obtained. An intermediate point was found where preparations at  $10^{-2}$  gm/cc were inactive but were active following the reactivation process. It seems probable, therefore, that two

simultaneous reactions occur, one reversible and the other irreversible. The former reaction probably reaches an equilibrium, while the latter one continues slowly, thus accounting for the long time required for complete inactivation. It was hoped that by varying the conditions the irreversible reaction could be minimized or stopped or the reversible one favored, but so far little success has been attained following the use of different concentrations of reactants, of different pH values, or of different aldehydes.

An increase in concentration of formaldehyde caused an increase in rate of inactivation, while an increase in hydrogen ion concentration to pH 6 resulted in a decrease in the rate of reaction. The results of attempts to reactivate samples that were inactivated under such conditions are included in Tables IV-VI. It may be noted that essentially the same degree of reactivation was obtained with such samples as was obtained in the other cases. When partially inactivated protein solutions were allowed to stand at 3° for several months, their activity increased only slightly. If they were then dialyzed at pH 3 for 3 days, their activity increased approximately 10-fold or to about that which was obtainable if they had been reactivated soon after inactivation.

It is evident that the amount of reactivation that may be secured with formolized tobacco mosaic virus protein is many times greater than can be attributed to experimental error, to the toxic action of inactive protein, or to the removal of any remaining traces of formaldehyde by the prolonged dialysis. It seemed desirable, therefore, to attempt to correlate the inactivation and subsequent reactivation with changes in the protein molecule.

*The Effect of Inactivation and of Reactivation on Amino Groups* — Formaldehyde-inactivated tobacco mosaic virus protein has been reported to contain only about 60 per cent of the amino nitrogen (Van Slyke) of active protein (2). This result has been confirmed and in addition it has been found that partially inactivated virus proteins contain intermediate amounts of amino nitrogen. However, because of the small amount of amino nitrogen, it was not possible to differentiate between the amino nitrogen contents of 1 per cent, 0.1 per cent, and 0.0 per cent active protein preparations. It is, therefore, not surprising that no changes were detected when the samples were

reactivated. It is quite possible that part of the formaldehyde is removed by the acetic acid used in the Van Slyke amino nitrogen procedure and the measurements that were made refer only to the protein irreversibly inactivated.

Since Duhère (18) found that formalized proteins did not react with ninhydrin at 37°, the possibility of using this reagent to detect changes in amino nitrogen was investigated. The procedure to be

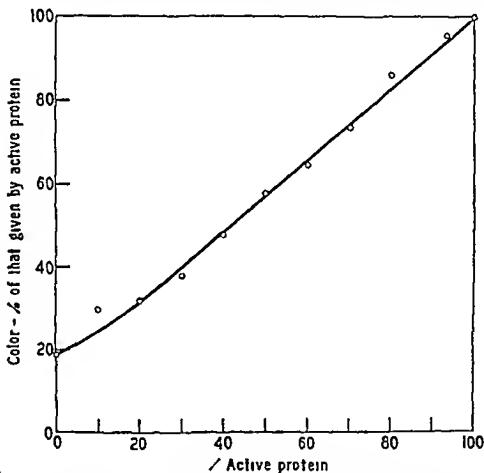


FIG. 2. The relative amounts of color obtained by means of ninhydrin with mixtures of active virus protein and virus protein completely inactivated by formaldehyde.

described was found most satisfactory. To 1 cc of a dialyzed virus solution (5 to 10 mg protein per cubic centimeter) in a 3 cc test tube were added 0.3 cc pyridine and 0.3 cc of a 2 per cent aqueous solution of ninhydrin. The solutions were mixed, stoppered, placed in an incubator at 37°C, and at the end of 20 hours they were diluted to 25 cc, allowed to stand ½ hour, and then compared by means of a photoelectric colorimeter. Fig. 2 presents the results of an experiment



in which mixtures consisting of active and of completely inactive virus proteins in different proportions were adjusted to the same total protein concentration and triplicate samples subjected to the above procedure. It may be seen that, considering the fact that the inactive protein gives some color, there is a good proportionality between the amount of color obtained and the proportion of active protein present. There was some variation in results, but changes in chromogenic power of 10 per cent were easily detectable when triplicate samples were used. Observations on the rate at which the samples developed perceptible amounts of color indicated that a given sample became colored in a shorter period of time than one containing 10 per cent less active protein.

Since the ninhydrin test seemed to be useful as a method of determining changes in amino nitrogen, it was used to determine the effect of inactivation and of reactivation on that grouping. In most of the experiments in which this test was used, the preparations were dialyzed at pH 3 for several days before the experiments were started. Partially inactivated preparations were then obtained and a portion of each reactivated as described above. The controls that were used consisted of portions of the original active virus protein preparations that were given exactly the same treatment as the samples, except that formaldehyde was not added. Triplicate samples were then treated with ninhydrin. The results of one of the more uniform experiments are shown in Fig 3. The color obtained with a given sample is expressed as per cent of that given by the control. Curve A represents the relative color intensities developed by samples following exposure to formaldehyde for the indicated periods of time, dialysis, and treatment with ninhydrin as described. Completely inactive preparations gave a small amount of color after 20 hours of incubation. Curve B represents the color intensity developed by the same samples after reactivation. In this experiment there was an increase in chromogenic power apparently due to the reactivation process. There was also a decrease in the chromogenic power of the control, and, if it can be assumed that the same change occurred in each sample, one may correct Curve B for such changes and thus obtain for the reactivated preparation Curve C. In the case of certain samples, dialysis at pH 3 for 3 days caused an increase in the color obtainable with the

controls. In such instances there were usually still greater changes in the same direction in the samples, thus still indicating an increase in chromogenic power due to the reactivation process. With but few exceptions, the above type of results was obtained. Invariably

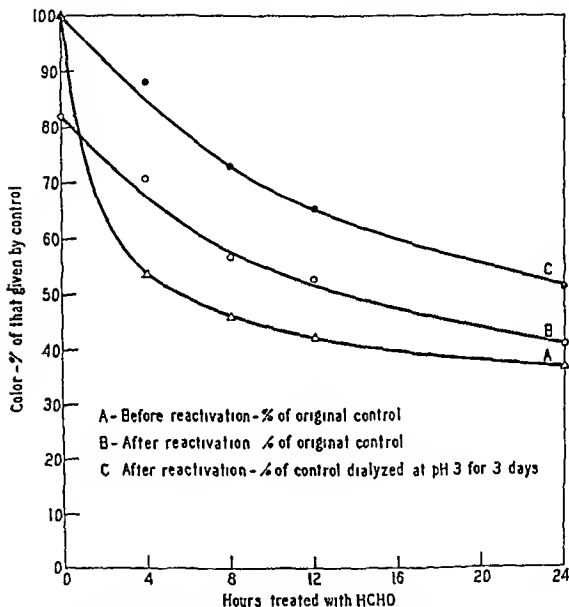


FIG. 3 The effect of inactivation and of reactivation on the amount of color obtainable with ninhydrin. Protein concentration of 5 mg/cc.

a gradual reduction in the amount of color obtainable with ninhydrin accompanied inactivation, but in a few cases, however, there was no indication of any increase due to reactivation.

Considered as a whole, the data indicate that the reactivation process results in an increase in the number of groups that react with

ninhydrin, presumably the amino groups. The variation in behavior of the controls and the occasional occurrence of negative results detract from the significance of the data, but a simultaneous increase in the color developed with ninhydrin and in activity occurred too consistently to be attributed to chance. As there seems to be no quantitative relationship between the amount of reactivation and the increase of color with ninhydrin, the results can only be considered as evidence that the reactivation process results in an increase in the groups that react with ninhydrin and that this is very probably due to the removal of formaldehyde from the amino groups.

*Effect of Inactivation and of Reactivation on the Groups that React with Folin's Reagent*—It was found that inactivation of the virus protein was accompanied by a decrease in the number of groups that reacted with Folin's phenol reagent. The effect was most noticeable when the color was allowed to develop at pH 7.5 to 8.0. The method used was essentially that of Herriott (19) except that sodium phosphate was used instead of the potassium salt, thus permitting the use of a stronger buffer without the formation of a precipitate. In a 50 cc flask were placed 10 cc of H<sub>2</sub>O and 10 cc of alkaline phosphate buffer ( $M/2$  Na<sub>2</sub>HPO<sub>4</sub> + NaOH, pH 10). 3 cc of Folin's phenol reagent (diluted 1 to 3) were then added, followed immediately by 0.5 cc of the dialyzed protein solution (5–10 mg/cc). This sequence was followed so that the protein at no time would be subjected to excessively alkaline or acid conditions, for denatured proteins may give higher values than native ones (20). The resultant reaction was at pH 7.7, varying somewhat with the particular buffer or phenol reagent. After standing at room temperature for 3 hours the samples were compared by means of a photoelectric colorimeter.

The same samples and controls that were used in the ninhydrin tests were also tested with Folin's phenol reagent. Results obtained with the latter reagent were more consistent than those obtained with ninhydrin. In every case a decrease in the color obtainable with Folin's reagent accompanied inactivation, and in only one experiment was there no indication of an increase in the number of groups that react with the reagent due to the reactivation process, and in that particular case very little reactivation was obtained. The reactivation process usually caused a decrease in the chromogenic power of the controls, yet in spite of this the reactivated samples practically always gave more color with the reagent than did the corresponding samples before reactivation. The results of a typical experiment are given in Fig. 4. Curve A represents the color developed when samples exposed to formaldehyde for the indicated periods of time were dialyzed and then tested with Folin's reagent. Curve B represents the color developed by the same samples after reactivation and is based on the same control. When the data are corrected for the effect of dialysis on the control, Curve C is obtained.

The consistency with which the above type of results was obtained indicates that formaldehyde reacts with some group or groups present on the protein molecule that also react with Folin's reagent and that the reaction is partially reversible. As far as it is known, the only

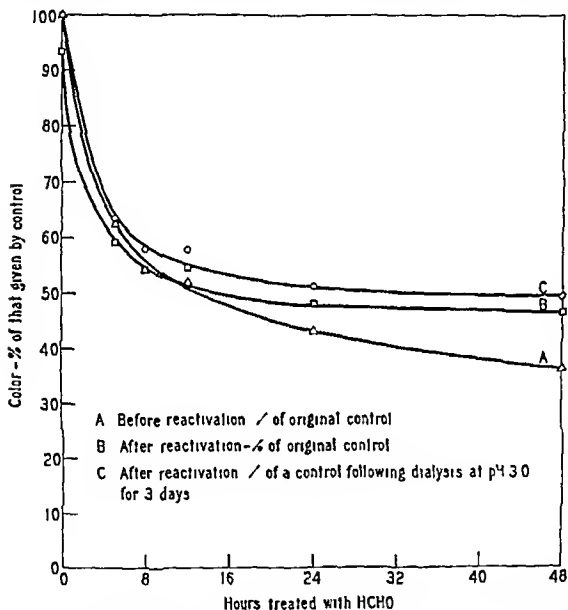


FIG. 4 The effect of inactivation and of reactivation on the amount of color obtainable with Folin's phenol reagent at pH 7.7

groups in proteins that react with that reagent are the phenolic groups of tyrosine (21), the indole nuclei of tryptophane (22), and sulfhydryl groups (23). Tobacco mosaic virus protein contains no sulfhydryl groups, but does contain appreciable quantities of the other two types of groupings. It, therefore, seemed desirable to determine the effect

of formaldehyde on the latter types when present in simple compounds. Solutions of 0.0025 M tryptophane, tyrosine, glycytyrosine, glycytryptophane, and indole propionic acid were treated with 2

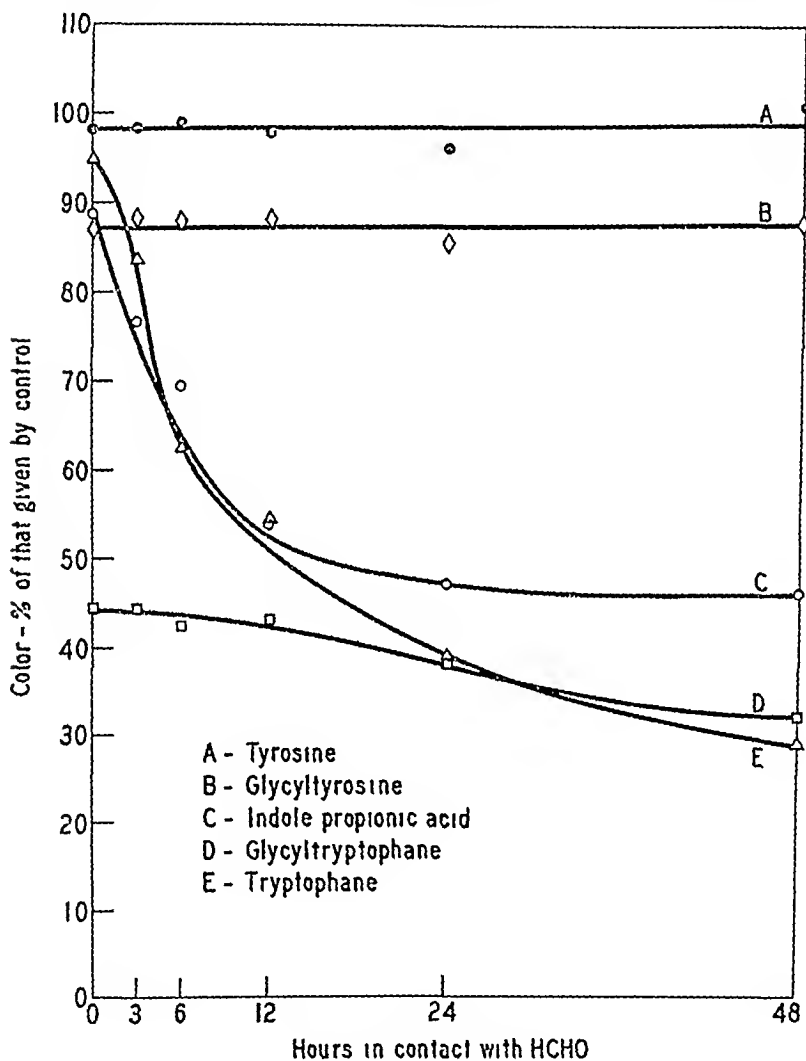


FIG 5 The effect of 2 per cent HCHO on certain indole derivatives at pH 7. The curves represent the relative amounts of color developed with Folin's phenol reagent at pH 7.7.

per cent formaldehyde in the presence of M/10 phosphate buffer at pH 7. At intervals samples were removed and tested with Folin's reagent, as already described. As formaldehyde gave no color with

the reagent, tests were made in the presence of the excess aldehyde. As may be seen from Fig 5 tyrosine did not react and glycyltyrosine gave only a small immediate reaction, but in the case of the compounds containing the indole nucleus there was an immediate reaction, especially in the case of the peptide, followed by a gradual decrease in the amount of color obtainable with the reagent. In the case of tryptophane a crystalline precipitate was formed. This is probably 3, 4, 5, 6 tetrahydro-4 carboline 5 carbonic acid, for Wadsworth and Pangborn (5) obtained such a compound under essentially similar conditions. When the crystalline compound was removed and washed, it gave some color with Folin's reagent at pH 7.7 but only a fraction of that obtainable with tryptophane itself. It will be seen from Fig 4 that no immediate reaction between formaldehyde and the virus protein is recorded. However, it is possible that such a reaction may actually have occurred, since the data for the virus protein were obtained using dialyzed samples, hence it was impossible to obtain a sample representing zero time for the reaction cannot be stopped instantaneously by dialysis. In experiments with the virus protein in which determinations were made without removal of the excess formaldehyde, there was an immediate decrease of about 15 per cent in the amount of color given with Folin's reagent at pH 7.7. At other points along the curve practically the same amount of color was obtained with a given sample, regardless of whether the formaldehyde was present or removed by dialysis. In view of the above results, it seems quite probable that the data obtained with the virus protein are due at least in part to the reaction of formaldehyde with the indole nuclei of the protein.

*Other Reactive Groups*—Other groups on the protein molecule that might react with formaldehyde are the imidazole ring of histidine (24) and the guanidine nucleus of arginine (25). Unpublished results obtained in this laboratory indicate that histidine is either absent or occurs in only small amounts in hydrolysates of the virus protein. Furthermore, qualitative tests for histidine on the intact protein molecule gave negative results. It was not deemed necessary, therefore, to study at this time the possible effect of formaldehyde on the imidazole ring. The virus protein was found to give a strong Sakaguchi test for arginine (26) and this test was not diminished by form

aldehyde treatment The test was carried out at a fairly strong alkaline reaction, and it may be that under such conditions formaldehyde was removed, although Eaton (25) found that when diphtheria toxin was treated with formaldehyde the Sakaguchi test was diminished Nothing is known concerning the action of formaldehyde on the virus nucleic acid However, it is quite probable that nucleic acid is not concerned in the chemical changes that have been measured The virus nucleic acid reacts slowly with nitrous acid and, under the conditions used, the gas given off by it in the Van Slyke determinations would not be measurable The virus nucleic acid gave only a trace of color with Folin's reagent at pH 7.7 and could not account for over 1 per cent of that given by the protein Addition of nucleic acid to formolized virus protein did not affect the color obtainable with ninhydrin

*The Action of Other Aldehydes*—The fact that formaldehyde reacts reversibly with more than one grouping of the virus protein molecule renders it difficult to determine the specific reaction causing loss of virus activity The action of other aldehydes on the virus protein was investigated, in view of the possibility that some of them might give but one type of reaction Acetaldehyde, propionaldehyde, and butyraldehyde gave essentially the same qualitative results as did formaldehyde Each caused a simultaneous decrease in virus activity, in groups that react with ninhydrin, and in groups that react with Folin's phenol reagent Reactivation was obtained in each case, although in general it was somewhat less than that obtained with formolized virus protein There was also less indication of chemical changes accompanying reactivation Benzaldehyde caused a gradual reduction in activity, but there were either no accompanying changes in the number of amino or other groups or such changes were quite small Samples of virus protein that were partially inactivated by benzaldehyde were reactivated to about half the extent of those that were inactivated by formaldehyde When a solution of the virus protein was treated with furfural, an insoluble precipitate was formed Considerable protein remained in solution, but such solutions were quite opalescent They possessed about 2 per cent of their original activity and gave less color with Folin's reagent or with ninhydrin than did the original active virus protein Dialysis at pH 3 for 3 days caused about a 10-fold increase in activity in most preparations

## DISCUSSION

The reactivation of formolized virus differs from that which has been secured following inactivation by safranine (27, 28) or with the salts of heavy metals (29, 30), for in the latter instances the complexes are insoluble and the possibility that they are non infectious because of toxicity has not been excluded. The fact that purified proteins were used and that tests were made in the absence of excess inactivating agent also distinguishes it from the reactivations reported by Zinsser and Seastone (31), Perdrau (32), and Schultz and Gebhardt (7). The results from tests with impure preparations could possibly be due to reactions in which the impurities play an important rôle. Tests for activity in which the excess inactivating agent has not been removed are open to question until it has been shown definitely that the agent has no effect on the susceptibility of the host. It is possible that an impurity present in very small amounts might account for the results obtained with the reactivation of formolized virus, but it seems quite unlikely in view of the constancy of the results, regardless of the method of purification used. Reactivation or recovery of spores or bacteria that have been attenuated with the salts of heavy metals (33-35) or with heat (36) have been reported, but in such cases no correlation has yet been established between chemical changes and changes in biological activity such as those described in this paper. It seems likely that in the case of microorganisms attenuation is due at least in part to an injury that the cell itself is able to overcome or to one that the cell can survive provided the poison is removed within a certain period of time. In the present experiments, however, there is no evidence of an "injury" to the virus due to formaldehyde that the virus itself can overcome. The introduction of the active virus remaining in partially inactivated preparations or of the virus in reactivated preparations into a host results in the production of active virus in a manner and at a rate that is indistinguishable from that following the introduction of ordinary virus. Furthermore, evidence has been presented that the formaldehyde can remain in combination with the virus protein for long periods of time without diminishing its ability to be reactivated. On the other hand, Gegenbauer (33) found that bacteria attenuated with  $\text{HgCl}_2$  could not be recovered by chemical treatment if they were allowed to remain in the dormant state for too long a period of time. The data reported



here are not regarded as evidence that the virus is either non-living or living but solely as evidence that virus activity is dependent upon a certain chemical structure that is found in the virus protein molecule

The data presented indicate that the inactivation of tobacco mosaic virus protein by formaldehyde is due to a blocking of active groups or at least to a destruction of a part of the structure essential for virus activity. The chemical reactions taking place may be quite complex. In view of the partial reversibility of the reaction, it appears quite likely that the formaldehyde combines with the protein. If inactivation were the result of a reduction, an oxidation, or a cleavage due to the formaldehyde, it seems quite unlikely that dialysis would bring about a reversal of the reaction. That formaldehyde is actually bound to the protein is indicated by the fact that the formolized proteins give a very strong Rosenheim-Acree test upon the addition of  $\text{H}_2\text{SO}_4$  and an oxidizing agent. It is possible, however, that the irreversible part of the reaction is due to one of the other types of reactions.

The similarities between the reactions occurring when the virus protein is treated with formaldehyde and those taking place between certain amino acids and formaldehyde under the same conditions are probably not without significance. It was shown that the indole nucleus, even though substituted in a variety of ways, reacted with formaldehyde under the conditions used, and it is reasonable to assume that it reacts with formaldehyde when present in a protein molecule.

The changes that occur in amino nitrogen in the reaction between formaldehyde and virus protein are similar in many respects to those that occur in reactions between formaldehyde and amino acids or other proteins. In the well known formol titration, equilibrium is reached quite rapidly, hence the reaction must differ from that causing inactivation of the virus, although of course the reaction may occur and be readily reversed in the plant. If amino acids or proteins are allowed to react with formaldehyde for several days, a gradual decrease of amino nitrogen (Van Slyke) occurs (4). The rate varies with the amino acid used, as does also the total amount reacting. It has been shown by Wadsworth and Pangborn (5) that this reaction with formaldehyde is partially reversible and that the reversibility decreases as the reaction between the amino acid and formaldehyde proceeds.

On the basis of their results, they suggested that there are at least three stages in the combination of formaldehyde with amino groups (1) a loosely associated molecular compound, (2) a labile chemical compound, possibly a methylene or hydroxymethyl derivative, and (3) a stable compound, probably formed by further reaction or rearrangement of the labile compound. Stages (1) and (2) were considered to be reversible, whereas stage (3) was not. This sequence of reactions serves to explain the present data very well. Thus, the results on the inactivation and the reactivation and the accompanying chemical changes could be explained by known chemical reactions. It is not possible at present to identify the specific reaction that causes the change in activity, but since these two reactions are associated so closely with the change in activity it seems quite likely that one or the other (or perhaps both) is responsible. It is concluded, therefore, that either the indole nuclei, amino groups, or both are part of the structure necessary for virus activity.

#### SUMMARY

A marked reactivation of tobacco mosaic virus protein that has been partially or completely inactivated by formaldehyde was obtained by dialysis at pH 3. The activity of partially inactivated virus proteins was generally increased about 10 fold by the reactivation process. It was also found possible to reactivate completely inactive preparations to an appreciable extent. It was shown that the inactivation and the subsequent reactivation cannot be explained by the toxicity of the formaldehyde or of the formolized protein or by aggregation.

Inactivation was accompanied by a decrease in amino groups as indicated by Van Slyke gasometric determinations and by colorimetric estimations using ninhydrin. Inactivation also causes a decrease in the number of groups that react with Folin's reagent at pH 7.7. The latter are probably the indole nuclei of tryptophane, for it was demonstrated that tryptophane, glycyltryptophane, and indole propionic acid react with formaldehyde in a similar manner, while tyrosine and glycytyrosine do not. Evidence that reactivation is accompanied by an increase in amino nitrogen and in groups that react with Folin's reagent was obtained by colorimetric estimation.

The demonstration that the addition of formaldehyde to the virus protein results in a simultaneous decrease of activity, of amino groups, and of groups that react with Folin's phenol reagent, and that under conditions favorable for the removal of formaldehyde the virus activity is regained and the number of such groups increases, indicates that certain of these groups play at least a partial rôle in the structure necessary for virus activity. These changes can best be interpreted on the basis of known chemical reactions and are considered as evidence that virus activity is a specific property of the protein.

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# CHEMICAL CHANGES IN THE ADDUCTOR MUSCLE OF THE CHELIPED OF THE CRAYFISH IN RELATION TO THE DOUBLE MOTOR INNERVATION

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Work from this laboratory has shown that a number of the striated muscles of the legs of the crayfish show a typical motor innervation (1) Such muscles are innervated by two motor fibers only, each causing a different type of contraction One of these, resulting from stimulation of the thicker fiber, is termed the "fast" contraction, while the other is called the "slow" Both anatomical evidence and physiological experiments indicate that each muscle fiber is innervated by these two axons and that both contractions occur in the same muscle fibers (2, 3)

The most striking difference between the two contractions is found in the adductor muscle of the cheliped of the crayfish In this case, a single impulse in the thicker motor fiber causes a twitch like contraction, and the system behaves in almost all respects as does a single motor unit of a vertebrate muscle single shocks of different strengths give twitches of a considerable strength and a constant height, and during tetanic contraction the action currents are all of the same magnitude In contrast to this, single shocks given to the thinner fiber produce no visible response in the muscle, but faradic stimulation causes action currents which grow in height and a contraction with a long latent period The action currents in this case are always much smaller than those of the fast contraction

The chemical changes occurring in the adductor muscle of the cheliped of the crayfish *Cambarus clarkii* during these two types of contraction were investigated in order to obtain evidence on two possible mechanisms by which the two contractions might occur in the same muscle fiber In the first place, two contractile substances

might be present, a "phasic" and a "tonic" one, analogous to those supposed by Botazzi (4) in the vertebrate striated muscle, or the same substance might contract in both cases, the difference between the contractions being due solely to differences in the transmission mechanism between the nerve impulse and the contractile substance.

Methods were devised for the rapid removal of the stimulated cheliped to liquid air and for the analysis of the frozen muscle tissue. The changes in phosphate and in lactic acid content were used as indices of the chemical changes occurring.

### *Methods*

Animals with paired normal chelipeds were used in this work. The chelipeds were cut from the animal at the ischiopodite and kept in the physiological solution described by van Harreveld (5). One cheliped served as a control for the experimental treatment of the other.

The motor nerve fiber giving the slow contraction, or that giving rise to the fast, was prepared in the meropodite in the manner described by van Harreveld and Wiersma (1). All of the motor and inhibitor fibers going to the other muscles in the cheliped were cut, as well as the unwanted motor fiber to the adductor muscle. Usually a small sensory bundle was kept intact to strengthen the bridge between the two thicker parts of the nerve bundle. The stimulating electrodes were always placed on the thicker proximal part of the nerve bundle in the meropodite.

Since it is impossible in preparing the fiber to avoid stimulation altogether, a rest period of between 45 and 120 minutes was allowed between the conclusion of preparation and the beginning of an experiment. The prepared cheliped was then fastened upon a wooden block fitted with flexible silver wire stimulating electrodes and with a rod for mounting upon a heavy support stand. The dactylopodite was tied to an isometric lever attached to the same stand. The electrodes were connected by flexible leads to a thyatron stimulator giving rectangular shocks of 0.5 $\sigma$  duration at various determined frequencies. The mechanical effect of the contraction resulting from the stimulus was recorded, and then the cheliped, still mounted on the block, was plunged into liquid air. Stimulation was continued during the period of removal to liquid air, this time (1-3 seconds) being included in the total time of stimulation. The control cheliped was also frozen at about the same time.

The frozen cheliped was opened, and the tissue of the adductor muscle was separated out (free of chitin and tendon) and dropped into liquid air. The tissue

fragments, together with a small amount of liquid air, were placed into the barrel of a small crusher, a greatly simplified form of that described by Graeser, Ginsberg and Friedemann (6). It is essential to keep the tissue fragments and the pellets obtained by crushing constantly cooled with liquid air since it was found that any thawing produces irregularities in the resting values between the muscles of paired, unstimulated chelipeds.

### *Phosphate Determination*

A method for the removal of proteins with 25 per cent trichloroacetic acid was developed, and the molybdate stannous chloride method of Kuttner and Cohen (7) was used for the determination of phosphate in the deproteinized solution. The values are reported as milligrams per cent of phosphorus.

The frozen muscle pellet (300–500 mg) is rapidly weighed on a torsion balance and dropped into 50 ml of ice cold 25 per cent trichloroacetic acid. The flask is stoppered and vigorously shaken for 15 seconds, and then ice cold water is added to make a total dilution to 250 ml, allowance being made for 80 per cent water content of the tissue. The diluted mixture is well shaken and set aside in an ice bath. Within 6 to 10 minutes aliquots are taken for colorimetric analysis. A red filter (Eastman Wratten filter 25A) is used in the colorimeter to avoid errors arising from the slight difference in color between standard and unknown.

It was found that, due to some property of the muscle proteins, the ordinary concentrations of 5 per cent or 10 per cent trichloroacetic acid failed to give complete precipitation, a flocculent precipitate appearing in the final color mixtures. When the pellets were shaken with 25 per cent trichloroacetic acid, however, the final colored solutions were clear. Before the colorimetric determination, the mixture had to be diluted to only 5 per cent trichloroacetic acid in order to avoid interference in the color development. The use of ice cold water was found to minimize the effect of hydrolysis of phosphagen and so to eliminate the necessity of an extrapolation to a zero time value. No catalysis of the hydrolysis of the phosphagen by acid-molybdate was observed.

Analyses carried through the complete procedure gave an average recovery of 99 per cent of added phosphate. The method of protein precipitation described has also been found suitable for dealing with *Astacus trowbridgi* and with *Pagurus ochotensis*.



*Lactic Acid Determination*

The protein precipitation procedure used was the modification of the zinc hydroxide method of Somogyi described by Graeser, Ginsberg, and Friedemann (6), with the exception that, since no sugar determinations were made, glucose was not added to the zinc sulfate-sulfuric acid solution. For the removal of bisulfite binding substances the copper-lime precipitation was used.

For the determination of lactic acid in the copper-lime filtrate a modification of the direct distillation procedure of Friedemann and Graeser (8) was made. Following the work of Avery, Kerr, and Ghantus (9), reduced volumes of sample and of reagents were used. The distillation apparatus was modelled after the micro-distillation apparatus of Lehnartz (10). The manganous sulfate-sulfuric acid reagent of Friedemann and Graeser was found to be as effective for the conditions adopted as the stronger reagent of Avery, Kerr, and Ghantus, and potassium permanganate was found satisfactory as the oxidizing agent.

An average recovery of 96 per cent was obtained with known lactic acid solutions carried through the entire procedure.

## RESULTS

*Examination of Possible Interfering Factors*

The suitability of the use of one cheliped to obtain the resting value for the other was investigated. In Table I are summarized the results of a number of experiments made to compare the resting value levels of phosphate and of lactic acid in the adductor muscles of paired unstimulated chelipeds. The difference between the chelipeds of one animal is small, so it is clear that one cheliped may be used as the control for the other.

There are several possibilities of production of phosphate and of lactic acid from stimuli other than those controlled in an experiment. The factors considered have been preparation of the nerve, rest in physiological solution, and the freezing of the cheliped in liquid air. The most important of these, in view of the unsymmetrical stimulation involved, is the preparation of the nerve bundle.

A series of experiments were performed in which the nerve bundle of one of a pair of chelipeds was given the maximum stimulation encountered in preparation. Both were then allowed to rest for the usual time, frozen, and analyzed. The same differences were found

TABLE I

*Difference in Phosphorus and Lactic Acid Values between the Adductor Muscles of Paired, Unstimulated Chelipeds*

Analysis for	Phosphorus			Lactic acid		
	Left muscle	Right muscle	Difference	Left muscle	Right muscle	Difference
Values found	51	57	$\pm 6$	19	26	$\pm 7$
	49	54	5	26	18	8
	40	39	1	34	29	5
	53	52	1	17	14	3
	55	53	2	19	20	1
	47	45	2	28	30	2
	51	49	2	25	30	5
	55	49	6	28	25	3
	51	48	3	14	15	1
	44	46	2	14	20	6
	47	44	3	17	22	5
	60	56	4	20	22	2
	58	60	2			
	51	51	0			
	60	58	2			
	59	59	0			
	44	48	4			
	51	53	2			
	48	53	5			
Mean value	51.1	51.2		21.8	22.2	
	$\pm$	$\pm$		$\pm$	$\pm$	
	1.2	1.2		1.9	1.5	
No. of values	19			12		

as are recorded in Table I for paired unstimulated chelipeds, therefore, the phosphate or lactic acid formed has disappeared on resting.

In a series of experiments in which one cheliped was dropped into liquid air immediately upon cutting from the animal, with the other being allowed to stand in physiological solution for periods up to 2 hours, there was found only a small

increase in phosphate and lactic acid in the latter. This amount is within the limits of variation noted for the resting values between paired unstimulated chelipeds.

It was observed that an unstimulated cheliped would sometimes have the dactylopodite in the closed position after liquid air treatment and sometimes not (the stimulated chelipeds were always closed). For many of the experiments on control values (Table I), a record was kept of this position of the dactylopodite. No correlation with the analytical results was found, and it was concluded that if the closing of an unstimulated cheliped is indicative of a real contraction, this contraction adds no measurable amount to the production of phosphate or of lactic acid. It is more likely that the closed position is indicative of some mechanical effect of freezing. Although Meyerhof and Lohmann (11) are of the opinion that freezing of the tissue causes a large breakdown of the phosphagen, the present work shows either that such an effect does not enter or that it is small and so uniform as not to enter into the present considerations. It may be that some thawing occurred in the work of Meyerhof and Lohmann, a condition conducive to high results.

#### *Stimulation of the Isolated Motor Axons at Determined Frequencies*

It is of special importance to note that at stimulation frequencies of 50 shocks per second for the fast contraction and at 200 for the slow, the resulting contractions are very much alike. The maximum tensions developed in different preparations are in the main very similar and are reached within 2 seconds, and both kinds of contractions have a similar fatigue. This is in accord with the findings of Wiersma and van Harreveld (3), who obtained evidence that in one cheliped these two contractions are almost identical in strength and in speed. In contrast to this, the slow contraction at 50 shocks per second shows a much more gradual rise in tension before the maximum is reached (about 15 seconds), and the maximum tension, though of the same order, is on the whole less. A few attempts were made to investigate the fast contractions arising at a stimulation frequency of 200 per second, but the very quick drop in tension after the maximum had been reached made impracticable their use for this investigation.

In Table II are summarized the results of the stimulation experiments. The string to the lever was cut as soon as the contraction began to drop from the maximum. In the case of the slow contractions obtained by stimulation at a frequency of 50 shocks per second,

fatigue is very much postponed, and it was possible to continue these for much longer periods than the fast at 50 or the slow at 200 shocks per second. It will be seen from the table that although the slow contraction at 50 shocks per second was of much longer duration, no significant increase in the amount of phosphate, and only a small increase of the lactic acid, was found. The increases in the amount of phosphate during contractions arising from stimulation of the fast system at 50 per second and of the slow at 200 for the same period were closely alike, both showing the same very definite rise above the base level. The lactic acid formation was also definitely larger than in the slow at 50 per second, but there was a difference which may be significant in that the slow at 200 seems to form less than the fast at 50 per second.

It is to be seen that practically all of the contractions of the fast at 50 and of the slow at 200 per second done for the lactic acid measurements are of a duration of less than 15 seconds. These experiments were performed at another time of the year than those on phosphate, and all preparations showed at this time a quicker fatigue. A similar difference is present in the slow contractions at 50 per second: in the lactic acid determinations, the average duration of contraction is 30 seconds, whereas the mean value for the phosphate determinations is 80 seconds.

No way could be found of correlating the tensions produced in the contractions of the muscles of the different animals with the chemical changes found. The nature of the attachment of the adductor muscle to the chitin and to the tendon makes extremely difficult a complete isolation of the liquid air frozen muscle. Thus the estimation of any value for the size of the muscle, a factor of great importance with regard to the total tension developed, was not possible.

A factor which is not clear from the tables presented is the relation between phosphate and lactic acid and the stimulation time. In order to show that such a relation is indeed present under our experimental conditions, the chemical changes after a determined number of twitches were measured. No preparation of the nerve fibers was necessary in these experiments (although carried out in a number of cases), since stimulation of the slow fiber at these frequencies has hardly any effect mechanically and (as is evident from the determina-

CHEMICAL CHANGES IN ADDUCTOR MUSCLE OF  
CRAYFISH CHELIPED

TABLE II

*Formation of Phosphate and of Lactic Acid during Slow and Fast Contractions*

Preparation		Analysis for								
Fiber	Frequency of stimulation (shocks per second)	Phosphate (as phosphorus)				Lactic acid				
		Stimulation time	Milligrams per cent found in			Stimulation time	Milligrams per cent found in			
			Control muscle	Stim muscle	Difference		Control muscle	Stim muscle	Difference	
Slow	50	sec				sec				
		51	49	43	-6	32	28	24	-4	
		62	58	55	-3	17	23	21	-2	
		122	59	57	-2	22	17	19	+2	
		32	58	61	+3	15	15	18	+3	
		92	49	53	+4	27	23	26	3	
		62	51	56	5	31	24	28	4	
		47	48	53	5	17	28	33	5	
		92	61	67	6	62	12	22	10	
		122	38	45	7	38	13	26	13	
		122	45	52	7	62	26	41	15	
						16	18	34	16	
						21	24	42	18	
Mean value			51 5±2 1	53 9±2 1			21 0±1 6	28 0±2 2		
Difference in mean values			2 4±3 0				7 0±2 7			
Fast	50	12	55	59	+4	8	17	24	+7	
		11	57	65	8	11	13	20	7	
		9	41	51	10	10	21	28	7	
		12	56	67	11	4	30	43	13	
		17	56	68	12	7	21	36	15	
		18	61	74	13	12	23	42	19	
		17	57	71	14	8	21	41	20	
		17	59	74	15	8	19	40	21	
		18	44	63	19	9	16	39	23	
		15	40	62	22	8	27	53	26	
		10	55	78	23					
		32	51	75	24					
		17	56	85	29					
Mean value			52 8±2 1	68 5±2 4			20 5±1 5	36 7±2 9		
Difference in mean values			14 7±3 0				16 2±3 3			

TABLE II—*Concluded*

Preparation		Analysis for								
Fiber	Frequency of stimulation (shocks per second)	Phosphate (as phosphorus)				Lactic acid				
		Stimulation time	Milligrams per cent found in			Stimulation time	Milligrams per cent found in			
			Control muscle	Stim muscle	Difference		Control muscle	Stim muscle	Difference	
Slow	200	sec				sec				
		17	54	64	10	7	17	18	1	
		17	58	70	12	8	12	16	4	
		17	56	69	13	10	16	21	5	
		17	41	60	19	18	17	24	7	
		16	60	82	22	8	18	30	12	
						11	18	31	13	
						11	26	41	15	
						9	22	39	17	
						7	31	49	18	
						6	21	47	26	
Mean value.			54 1±3 3	69 7±3 4			19 9±1 6	31 7±3 3		
Difference in mean values.			15 6±4 8					11 8±3 7		

TABLE III

*Phosphate and Lactic Acid Formation on Single Shock Stimulation*  
(Frequency = 1, 3, 6 or 12½ shocks per second)

Total number of twitches	Milligrams per cent increase in			
	Phosphorus		Lactic acid	
	No of values	Average value	No of values	Average value
15- 50	3	7	9	7
50-100	8	11	7	8
100-150	2	13	4	12
150-200	3	23	2	20
200-400	3	23	—	—

tions upon slow preparations at 50 per second) certainly causes no appreciable chemical changes. The results are given in Table III. It will be seen that the production of phosphate gradually rises with

an increasing number of twitches and that the lactic acid formation is somewhat similar, although not so clearly demonstrated as that of the phosphate

### *Phosphagen Phosphate*

Since the amount of phosphate found in the stimulation experiments was, on the average, no more than 16 mg per cent phosphorus (highest value, 29 mg per cent) above the resting level, it was of interest to

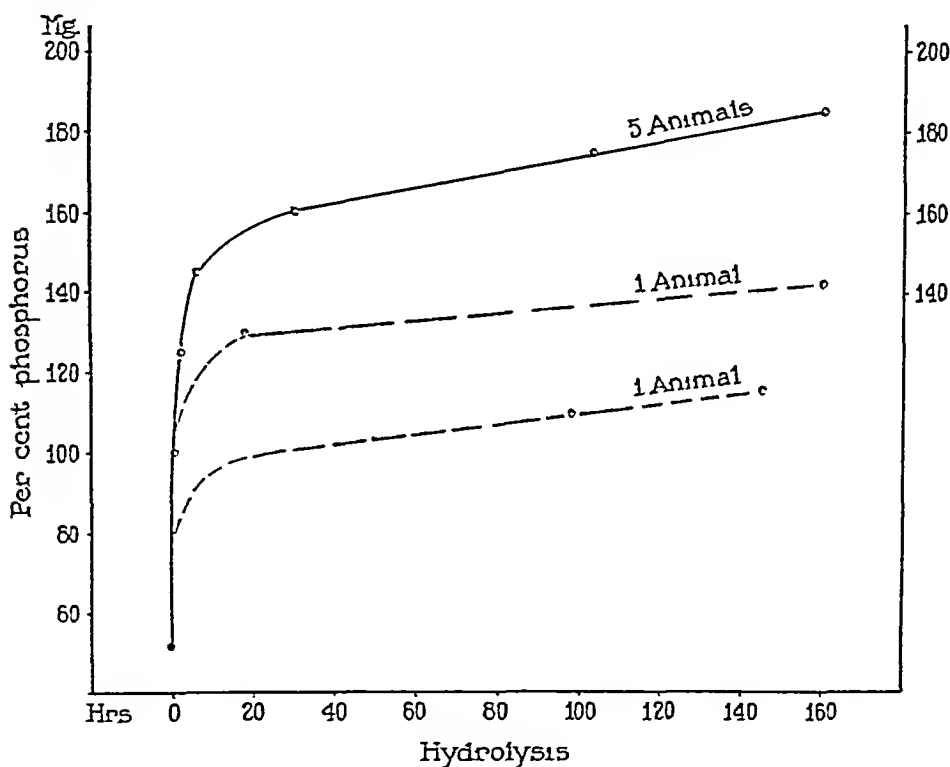


FIG 1 Hydrolysis of phosphate compounds in the adductor muscle

determine the fraction of the total available phosphagen represented by this amount. A number of experiments were made in which pairs of chelipeds were frozen in liquid air, with or without stimulation of one of the pair. The initial phosphate was determined, and the mixture of tissue and 5 per cent trichloroacetic acid was left standing at 35°C, determinations being made at intervals.

The results are plotted in Fig 1, the initial portion of the curve for one experiment being shown in detail in Fig 2

In the group of five animals giving closely concordant data, it will be seen that during the first 2 hours the hydrolysis proceeds rapidly a level of 130-135 mg per cent phosphorus being reached. The rate then decreases, and after about 30 hours (level of 160 mg per cent) a new, much slower rate is followed. At the end of 160 hours the final levels for the five pairs of chelipeds are 198, 197, 181, 176, 189, 185, 176, 174, 184, 184 the average is 185 mg per cent

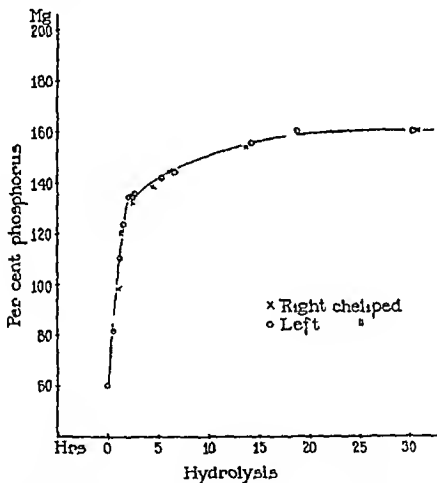


FIG 2 Production of phosphate on hydrolysis of the tissue from the paired claws of one animal

If the hydrolysis during the first 2 hours is considered as being that of the phosphagen and the remainder is assumed to be that of other more or less readily hydrolyzable phosphate compounds, the representative phosphagen level should be about 140 mg per cent. With a resting level of phosphate at 50 mg per cent phosphorus, the amount of available phosphagen is in the order of 90 mg per cent phosphorus



Thus, on the average, less than one-fifth of the available phosphagen was found hydrolyzed on contraction, at the most one-third

Experiments in which one of the pair of chelipeds was stimulated showed at the beginning of the hydrolysis the accustomed difference in the phosphorus level, but this difference had disappeared at the end of the first 2 hour period

Of the two deviating animals, one had a phosphagen level at about 110 mg per cent and a final level at 142 mg per cent phosphorus, the other, only 90 and 116 mg per cent respectively. Also in these cases, the paired chelipeds gave closely similar figures

#### DISCUSSION

The present investigation was undertaken in order to compare the chemical changes in the fast and the slow contractions and to correlate this information, if possible, with existing physiological data. The results obtained clearly show that the chemical changes involved in the two types of contraction are of the same order if the mechanical effects are comparable, and that they are distinctly different if the stimulation is such as to produce quite different mechanical effects. Since equivalent changes accompany equivalent action, it is necessary to assume that the two types of contraction do not differ in the mechanism of the chemical changes involved, which is strongly indicative of the presence of only one contractile substance.

The evidence obtained thus strengthens the conclusions arrived at by anatomical and physiological experiments (2, 3), namely, that each muscle fiber is innervated by both the fast and the slow axon and that both contractions occur in the same muscle fiber. The difference between the two contractions then lies in the modes of transmission at the neuromuscular junction.

For the present considerations more emphasis has been placed upon the phosphate values, for it is generally considered that the phosphate stands in a more directly integral position in the cycle of chemical changes in muscle than does the lactic acid. That only a fraction of the available phosphagen is found broken down to phosphate after contraction is of significance towards understanding the individual variations observed between animals. This fraction is probably more

apparent than real, its exact magnitude depending upon the balance between breakdown and resynthesis. Also, the supply of total phosphate and of available phosphagen is variable between animals.

The fact that no catalysis of the hydrolysis of the phosphagen in acid molybdate solution was observed in the course of the phosphate determinations indicates that the phosphagen of *Cambarus clarkii* is arginine phosphoric acid rather than the creatine compound. This conclusion is substantiated by the results of previous investigators (Kutscher (12), Eggleton and Eggleton (13), Meyerhof and Lohmann (14), Schutze (15)) on the isolation of the phosphagen of crustaceans.

The resting value for phosphate of Schutze on *Astacus fluminalis* (about 90 mg per cent phosphorus) is higher than that found in the present work on *Cambarus clarkii*, as are his maximal values (240 mg per cent). The high resting value probably is due to injury of the muscle tissue during preparation for analysis. Meyerhof and Lohmann (11) give a much lower resting value (about 35 mg per cent) for *Astacus*, but their maximal values are only about 100 mg per cent. The discrepancy in the breakdown phosphate values no doubt lies in the conditions of the hydrolysis and that the values of Meyerhof and Lohmann are lower than those of the present work may be due to a difference between *Astacus* and *Cambarus*. In this connection it is of interest that the available phosphagen for the latter amounts to 65 per cent of the sum of resting phosphate plus that from phosphagen, a figure in agreement with the 60-75 per cent noted by Meyerhof and Lohmann for *Astacus*.

The very high resting lactic acid values of Schutze (average 322 mg per cent for 9 values) are not in accord with the present data. However, the few values given by Meyerhof and Lohmann are of the same order as the present ones, and it may be that the analytical method used by Schutze gave apparent high results.

#### SUMMARY

An investigation has been made of the phosphate and lactic acid changes in the adductor muscle of the cheliped of the crayfish *Cambarus clarkii* upon stimulation of the isolated axons for the fast and slow contractions at determined frequencies. The data obtained point to the following conclusions:

1. When the mechanical effects of the two types of contraction are the same, the chemical changes are of the same order. If the mechanical effects are different, the chemical changes likewise are not equivalent. This is especially to be seen in the case of stimulation at 50 shocks per second: a slowly rising, long continued, strong slow contrac

tion takes place with no apparent change in the phosphate content, a quickly rising fast contraction occurs with a large increase in the phosphate

2 Since equivalent chemical changes accompany equivalent mechanical action, the two types of contraction do not differ in the essential mechanism of the chemical changes involved, and only one type of contractile substance is present

3 Even when a contraction has taken place to the maximum extent obtainable, only enough phosphate is found to correspond to one-fifth to one-third of the available phosphagen

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# FORMATION OF NEW CRYSTALLINE ENZYMES FROM CHYMOTRYPSIN

## ISOLATION OF BETA AND GAMMA CHYMOTRYPSIN

By M. KUNITZ

*(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)*

(Accepted for publication August 2, 1938)

The isolation of crystalline chymotrypsinogen from fresh beef pancreas and its conversion into chymotrypsin by trypsin has been described in former publications (1).

Chymotrypsin in solution undergoes a gradual irreversible transformation into new enzymes, two of which have now been isolated in pure crystalline form. The new enzymes, called beta ( $\beta$ ) and gamma ( $\gamma$ ) chymotrypsins, are proteins and are enzymatically indistinguishable from the original chymotrypsin but differ from it in molecular weight, crystalline form, stability in acid or alkali, solubility, etc. The process of the irreversible transformation of chymotrypsin into the new enzymes, as well as a description of the methods of their isolation in pure crystalline form and a description of certain of their properties, are presented in this paper.

The procedure for preparing chymotrypsin consists essentially in adding a small amount of trypsin to a concentrated solution of pure chymotrypsinogen of pH 7.6 and allowing the solution to stand at 5°C for 24 hours. During this time complete conversion into chymotrypsin takes place. The pH of the solution is then adjusted to 4.0 and the chymotrypsin is salted out by means of ammonium sulfate. The amorphous precipitate is redissolved in a very small amount of 0.01 normal sulfuric acid and left at 20°C. A heavy crop of rhombohedral crystals of chymotrypsin is usually obtained within 24 hours. The yield is about 50 per cent of the total chymotrypsinogen protein used.

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A solution of crystalline chymotrypsin on recrystallization gives

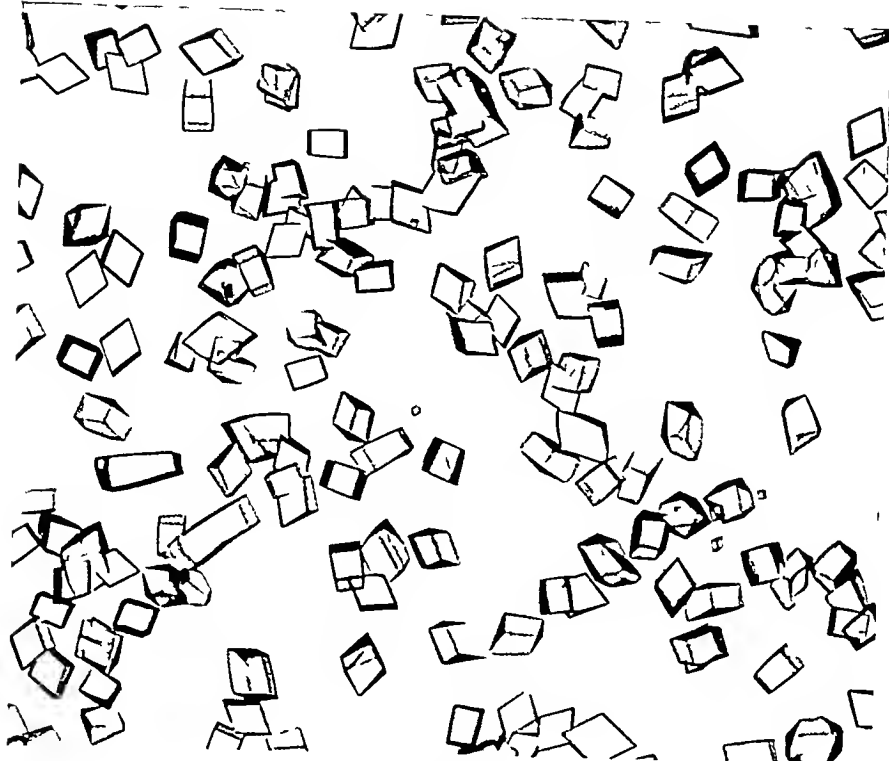


FIG 1a

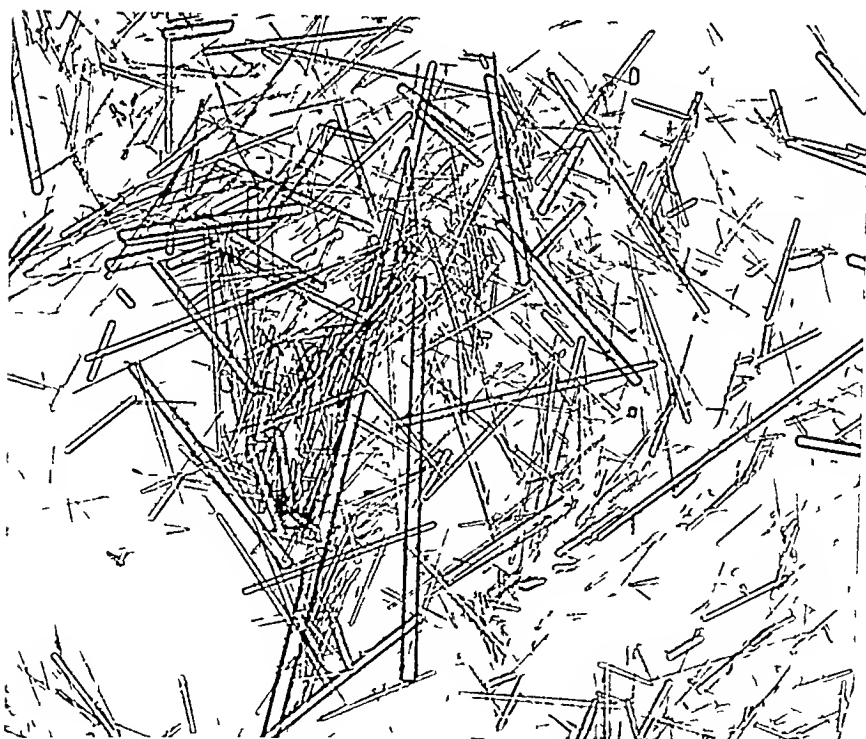


FIG 1b

FIG 1 Chymotrypsin crystals 1a, crystallized at pH 4.0  $\times 123$  1b, crystallized at pH 5.6  $\times 153$

rise either to rhombohedrons (Fig 1*a*) or to long prisms (Fig 1*b*), depending on the pH of the solution. In the range of pH 4.0-5.0 the solution yields rhombohedral crystals while at pH 5.2-6.0 prisms appear. The prisms, when recrystallized from a solution of pH less than 5.0 yield rhombohedrons and *vice versa*, thus proving that both forms of crystals are reversible polymorphs of one and the same substance—chymotrypsin.

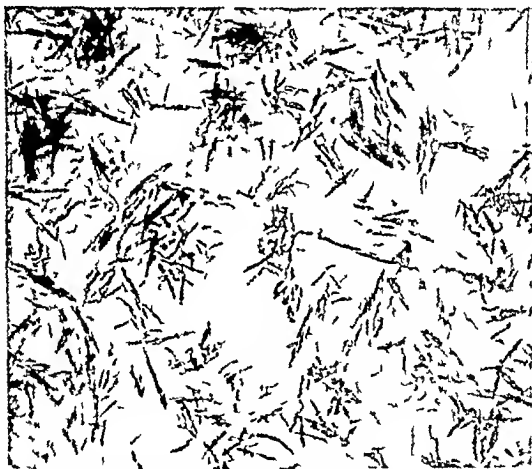


FIG. 2. Needle shaped crystals consisting of a solid solution of beta gamma and inert protein crystallized at pH 4.0.  $\times 300$

The mother liquor of the first crystals of chymotrypsin still contains about 50 per cent of the original proteolytic activity in solution and when kept at room temperature at pH 4.0 a protein crystallizes in the form of fine needle shaped crystals (Fig. 2) different from the rhombohedrons or the long prisms of chymotrypsin (2).

The rate of formation of the fine needle crystals is very slow but once started the mass of crystals continues to increase for weeks until



the solution becomes a semi-solid mass of fine crystals. The new protein crystals differ from those of chymotrypsin not only in form but also in solubility. They still possess the same type of enzymatic activity as that of chymotrypsin although there is a quantitative difference, the activity per unit weight of the new protein being about one-half that of chymotrypsin. On recrystallization at pH 4.0 the new protein always gives rise to the same fine needle-shaped crystals and all attempts to make it crystallize in the form of typical chymotrypsin rhombohedrons or prisms failed. It thus became evident that the new crystalline protein, unlike the prisms or the rhombohedrons, is not a polymorphic form of chymotrypsin.

*Origin of the New Crystalline Protein* —The new crystalline protein was first obtained from the mother liquor of the chymotrypsin crystallization and the possibility existed that the protein was present in the original crude extract. It was found, however, that the material could be obtained from repeatedly recrystallized chymotrypsin which in turn had been prepared from repeatedly recrystallized chymotrypsinogen. Solubility measurements (3) have shown that chymotrypsinogen and chymotrypsin are pure proteins so that the new protein must be formed from chymotrypsin and is not present in the original extract. The method of forming the new protein from pure chymotrypsin consists in allowing a concentrated solution of recrystallized chymotrypsin to stand at pH 7.6 and 5°C for about 3 weeks. An appreciable loss of activity takes place under these conditions without any significant loss of protein. The solution, when brought to pH 4.0 and with proper adjustment of the ammonium sulfate concentration, yields at room temperature the new protein crystals, the yield being 50–75 per cent of the total protein.

On repeated recrystallization the new protein retains the same crystalline form and also constant specific activity, which makes it appear that the new protein is a pure substance of constant composition. Measurement of the solubility of the new protein crystals in dilute ammonium sulfate solution in the presence of an excess of the solid phase shows, however, that the solubility of the crystals is not independent of the total amount of the excess crystals in suspension but continues to increase with the increase in the amount of crystals suspended. This does not accord with the phase rule criterion of a

pure substance. The plotted solubility curve (Fig. 3) showing the relation between the amount of the new protein dissolved and the total amount of protein in suspension corresponds to the theoretical solubility curve of "mixed crystals" consisting of a solid solution of two or more substances (3).

Further investigation has proved that the new protein crystals actually consist of a solid solution of several proteins two of which have been isolated in pure crystalline form and found to be active proteolytic enzymes of the chymotrypsin type. These two crystalline enzymes named beta chymotrypsin and gamma chymotrypsin can be easily isolated from a solution of the mixed crystals in 0.4 saturated ammonium sulfate by means of fractional crystallization at various

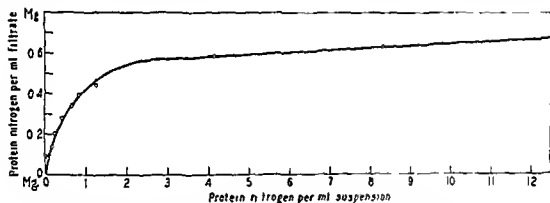


FIG. 3. Solubility at 10°C of solid solution crystals in 0.4 saturated ammonium sulfate pH 4.0 in the presence of increasing quantities of solid phase.

pH. At pH 5.0 or higher the solution yields on standing at 20°C large polyhedral crystals of gamma chymotrypsin, bipyramidal in form (Fig. 4). The filtrate from the gamma crystals when brought to pH 4.2 yields fine needle crystals of beta chymotrypsin. The beta crystals generally appear in the form of leafy rosettes and when very small, resemble the mixed crystals. The beta crystals have a tendency to form solid solutions with other proteins and it was found that the first beta crystals although practically free of gamma protein still contain in solid solution a great deal of inert protein which cannot be removed from the beta crystals by repeated recrystallization. It can, however, be easily removed by incubating a solution of impure beta crystals of pH 7.6 for 30 minutes at 37°C. The inert protein is then completely digested by the excess of active proteolytic

beta protein The latter can then be crystallized in a pure state (Fig 5)

Methods have also been developed whereby chymotrypsin can be readily changed directly into either one of the new crystalline proteins. Thus, if a concentrated solution of chymotrypsin crystals in dilute ammonium sulfate of pH 8.0 is kept at 35°C the chymotrypsin protein is completely changed within 90 minutes into gamma protein



FIG 4 Crystals of gamma chymotrypsin crystallized at pH 5.6  $\times 155$

On adjusting the pH of the solution to 5.6 large polyhedral bipyramidal gamma crystals rapidly appear. On the other hand if the change is allowed to proceed at a lower pH or lower temperature then either beta crystals or the mixed crystals of the solid solution are obtained.

On repeated recrystallization each enzyme continues to yield its own characteristic crystals. Chymotrypsin cannot be obtained again from either beta or gamma chymotrypsin. The change from

chymotrypsin to the new protein crystals is apparently an irreversible process.

The new enzymes differ from each other as well as from the original chymotrypsin not only in crystalline form but in a number of other respects such as molecular weight, stability in acid or alkali, degree of denaturation by urea, etc. Most striking are the quantitative differences in the solubilities of the three crystalline proteins in aque-

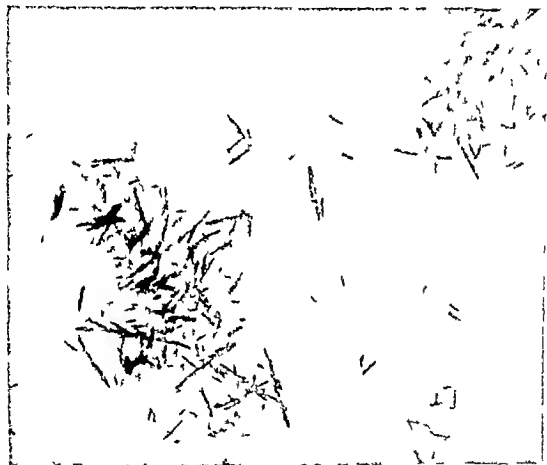


FIG. 5 Crystals of beta chymotrypsin crystallized at pH 4.0  $\times 315$

ous ammonium sulfate solutions. Thus at pH 4.0 gamma is six times as soluble as beta and about eight times as soluble as chymotrypsin. A solution saturated with crystals of chymotrypsin continues to dissolve crystals of beta and *vice versa*, the total amount of protein dissolved being equal to the sum of the individual solubilities. The same holds true for a mixture of gamma and chymotrypsin crystals when mixed at pH 5.5. On the other hand, crystals of gamma form a solid solution with either beta or chymotrypsin crystals when mixed

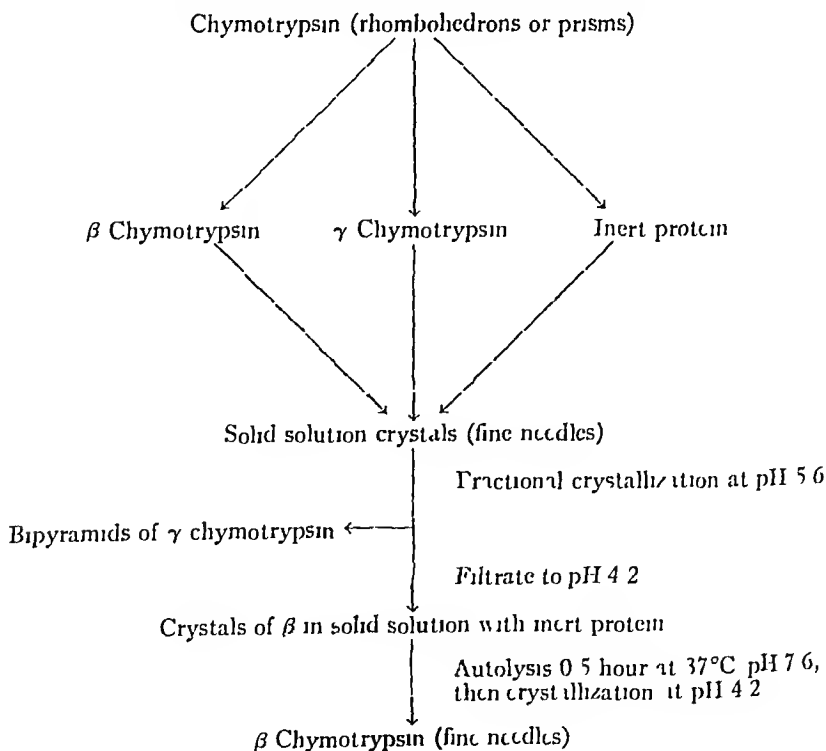
at pH 4.0, hence the solubility of gamma at pH 4.0 is affected by the presence of either one of the other protein crystals and *vice versa*

The phase rule solubility curves for the three crystalline proteins, unlike the solubility curve for the solid solution crystals, are typical of substances which contain only a small fraction of impurities

The three enzymes, while strikingly different in many respects, are, nevertheless, chemically and enzymatically very similar. No significant differences have been found in the elementary composition nor in the free amino nitrogen and tyrosine-tryptophane content of the three proteins, and no marked quantitative differences in the rate and extent of digestion of various proteins and artificial substrates have been observed. The formation of the new enzymes from chymotrypsin is apparently due to a slight hydrolysis of the chymotrypsin molecule as evidenced by the loss of protein during the process of formation of the new enzymes as well as by the fact that their molecular weights are lower than that of the original chymotrypsin.

In the following text as well as in the figures and tables chymotrypsin is frequently designated for brevity's sake by the Greek letter alpha ( $\alpha$ )

*Schematic Presentation of Formation of  $\beta$  and  $\gamma$  Protein from Chymotrypsin*



## EXPERIMENTAL RESULTS

*I Isolation of Beta and Gamma Chymotrypsins**1 The Formation of New Enzymes from Chymotrypsin at 5°C and pH 7.6*

*Experimental Procedure*—20 gm. of semi dry paste of crystals of chymotrypsin three times recrystallized from 0.4 saturated ammonium sulfate pH 4.0 was suspended in 48 ml. ice cold water and 12 ml.  $M/2$  phosphate buffer pH 8.0 was then added. This brought about rapid solution of the crystals. The pH of the solution was then adjusted to 7.6 by means of a few drops of 5  $N$  sodium hydroxide and stored at 5°C. Samples of 1 ml. were taken at various times and diluted with  $M/400$  hydrochloric acid to proper concentrations for activity and protein nitrogen determinations. At the same time samples of 3 ml. were taken for crystallization tests. These were mixed in test tubes with 2 ml. of saturated ammonium sulfate and after adjustment of pH to 4.2 by means of several drops of  $N/1$  sulfuric acid were left at 20–25°C for crystallization. The crystal suspensions were filtered after 3 days through Whatman's No. 42 paper and the clear filtrates were analyzed for protein nitrogen. The percentage yield of crystallized protein was then calculated.

The results of the various determinations are given graphically in Fig. 6 which shows that the process of transformation of chymotrypsin at 5°C and pH 7.6 consists of three successive stages. During the first stage, which includes approximately the first 2 or 3 days, a rapid formation of inert protein takes place as evidenced by the rapid decrease in the activity of the solution accompanied by a relatively small loss in protein. The inert protein formed interferes considerably with the crystallization of the active protein left. The solution, adjusted to the proper pH and ammonium sulfate concentration, still continues to yield crystals of normal chymotrypsin rhombohedrons, but the percentage of protein crystallized diminishes rapidly from day to day until the second stage is reached which includes the period between the third and fifth days when only a slight precipitate of spheroid granules mixed with a few rhombohedrons and fine needles appear in the crystallization samples. Finally during the third stage which extends for several weeks after the fifth day, the solution begins to yield increasing amounts of crystals again. The crystals, however, consist not of chymotrypsin rhombohedrons but entirely of fan shaped bundles of fine needles consisting of a solid solution of beta and gamma chymotrypsins and inert protein.

The yield of the needle crystals increases rapidly from day to day for a period of about 10 days until it reaches a maximum of nearly 70 per cent of the total protein present in solution. The crystalliza-

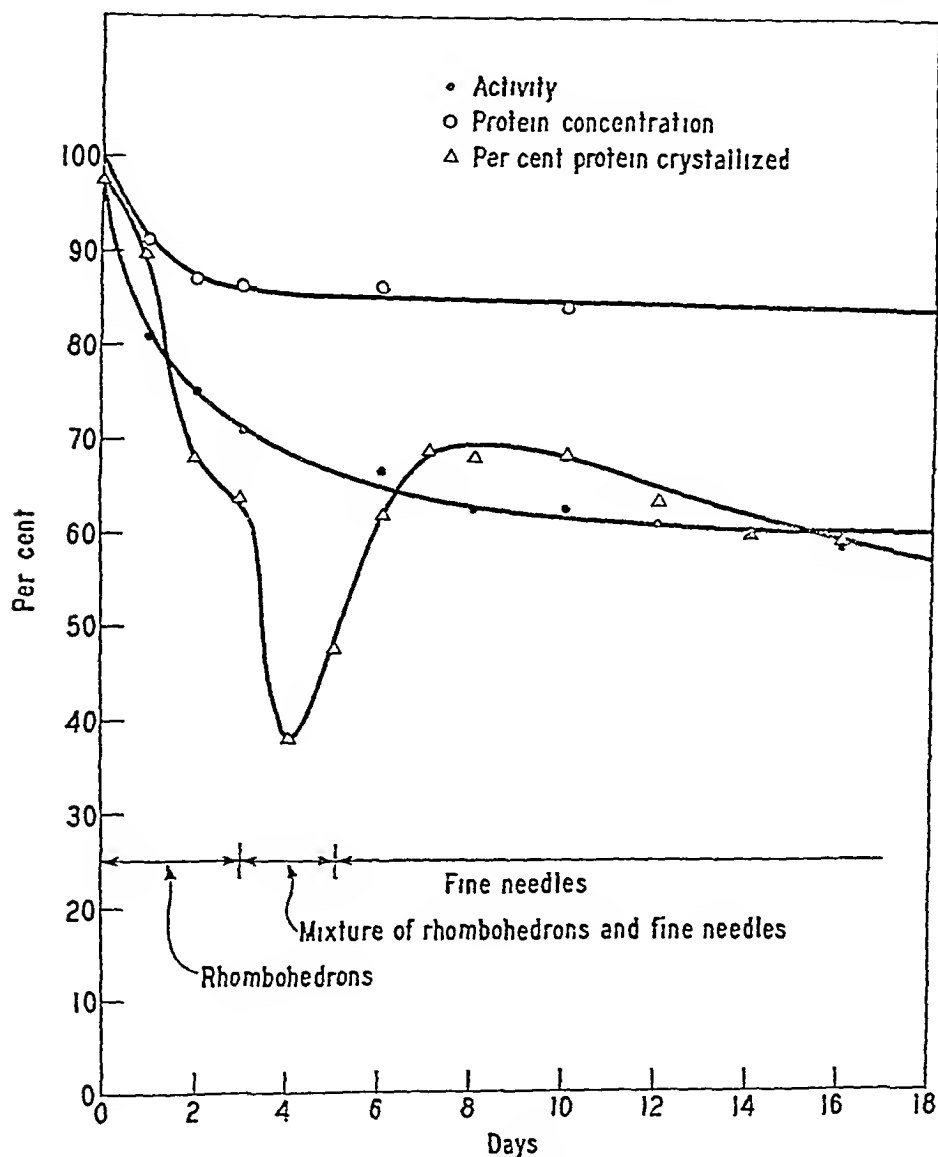


FIG 6 Formation of new proteins from chymotrypsin at pH 7.6 and 5°C

tion samples appear then as thick gels of fine crystals. On further standing autolysis takes place and the yield of crystals gradually diminishes.

The inert protein formed during the first phase of the process does not interfere with the crystallization of the new crystals since it combines with them to form a solid solution

### *2 Isolation of Gamma Crystals from the "Solid Solution" Crystals*

It has been mentioned that the needle crystals on recrystallization at pH 4.2 always retain the same needle form of crystals. If however the pH of the recrystallization mixture is adjusted to 5.2-6.0 then bipyramidal crystals of gamma chymotrypsin appear. The gamma crystals on recrystallization even at pH 4.2 yield only bipyramids and all attempts to change the gamma crystals to the original solid solution needles have so far failed.

*Experimental Procedure*—10 gm. of needle solid solution crystals was dissolved in 30 ml. water and the pH was adjusted to 5.6 by means of a few drops of 5 N sodium hydroxide. 20 ml. saturated ammonium sulfate was then added and the clear solution was left at 20°C.

Bipyramidal crystals gradually appeared and grew rapidly until a heavy sediment of crystals was formed within 24 hours. Crystallization was allowed to proceed for 2 days. The crystals were removed by filtration with suction on hardened paper and recrystallized several times in 0.4 saturated ammonium sulfate at pH 5.6 and finally at pH 4.2. The specific activity of the gamma crystals was about the same as that of pure chymotrypsin.

### *3 Isolation of Beta Crystals*

The filtrate from the gamma crystals when adjusted to pH 4.2 yields fine needle crystals similar to those of the original solid solution crystals but of a lower specific activity. They were named 'crude' beta crystals. The low specific activity is due to the presence of inert protein which forms a solid solution with the needle crystals. All attempts to remove the inert protein by means of fractional crystallization or repeated recrystallization were unsuccessful. It can be easily removed however by means of autolysis at 37°C.

*Experimental Procedure*—10 gm. of three times recrystallized crude beta crystal cake was dissolved in 250 ml. water and 10 ml. 0.4 M borate buffer pH 9.0 was added. The mixture, pH 7.6 was rapidly warmed to 37°C and left for 1 hour in a water bath at 37°C. Samples were taken at 15 minute intervals for measurements of activity and protein content. At the end of the hour the solution was cooled to 20°C and the pH was adjusted to 4.0. Solid ammonium sulfate was added to bring the solution to 0.4 saturation. This gave rise to a slight amorphous precipitate which was filtered off with the aid of about 1 gm. Standard Super Cel. The clear filtrate was brought with solid ammonium sulfate to 0.7 saturation. The formed precipitate was then filtered on hardened paper. The semi-dry amorphous precipitate of about 7 gm. was then dissolved in 21 ml. water and 14 ml. saturated ammonium sulfate was added. The pH was adjusted to 4.2. Crystals of fine rhomboid plates of pure beta gradually appeared. The specific activity of the pure beta crystals was about the same as that of chymotrypsin.



Fig 7 shows that the specific activity of the solution of crude beta trypsin is gradually increased because of the digestion of the inert protein by the active beta protein

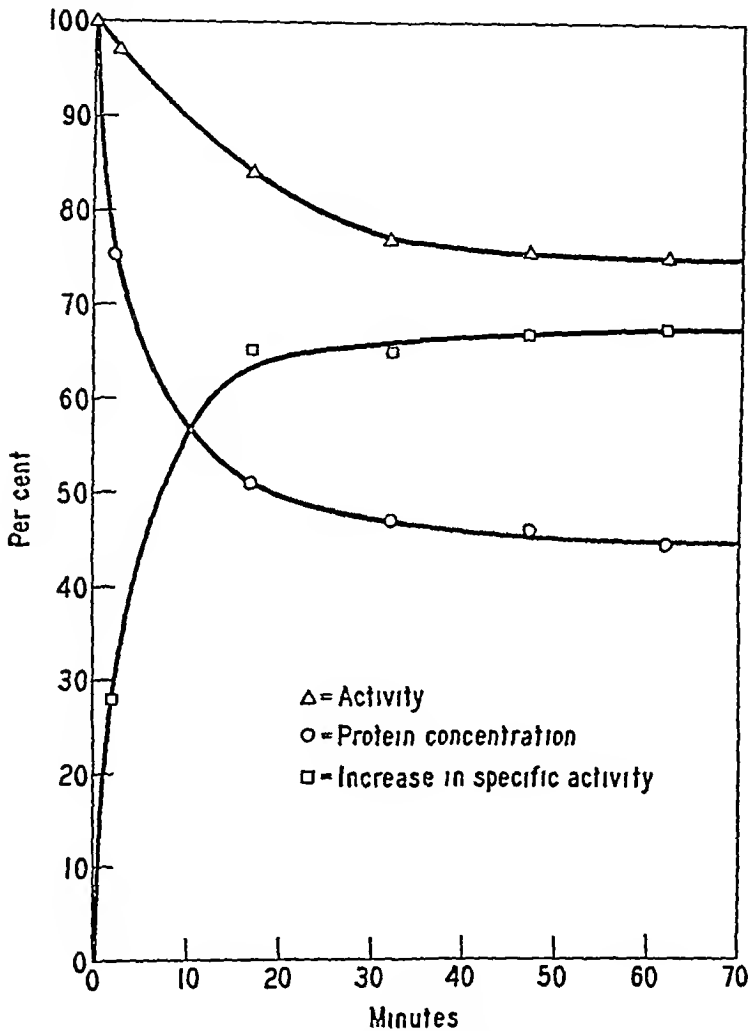


FIG 7 Removal of inert protein from beta chymotrypsin by hydrolysis at pH 7.6 and 37°C. The inert protein is rapidly digested by the active beta protein.

#### 4 Formation of Gamma Chymotrypsin from Chymotrypsin at 35°C and pH 8.4

The formation of the new enzymes from chymotrypsin at 5°C and pH 7.6 as described in section 1 is a slow process. The change occurs faster if the chymotrypsin is exposed to higher temperatures,

but the composition of the new materials yielded at various temperatures is not the same. It was found that when a concentrated solution of chymotrypsin is kept at 35°C and pH 8.4 a very rapid change

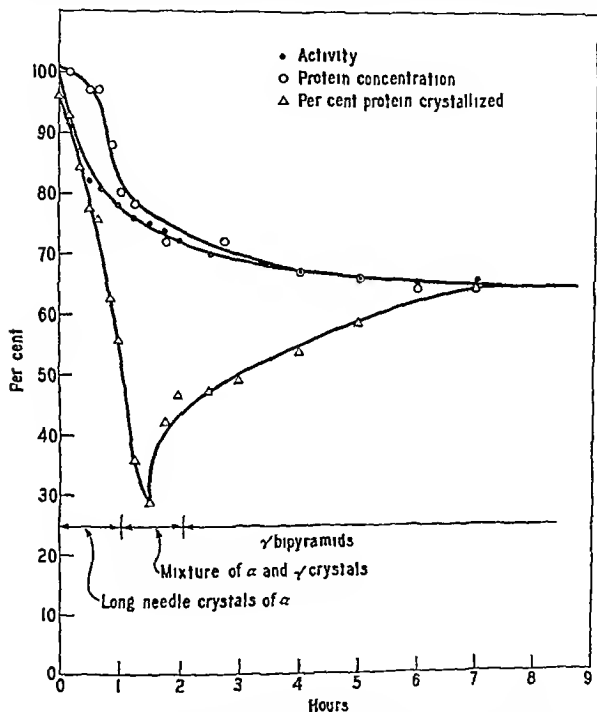


FIG 8 Formation of gamma from alpha chymotrypsin at pH 8.4 and 35°C

occurs from chymotrypsin into gamma chymotrypsin, without formation of any beta or inert protein. The last, if formed at all, is immediately digested under these conditions.

*Experimental Procedure* —A mixture was made of 16 gm of six times recrystallized chymotrypsin crystal cake plus 24 ml water plus 24 ml 0.4 M borate pH 9.0. The final pH of the solution was about 8.4. The solution was left at 35°C. Samples of 0.2 ml were taken at various times and diluted with N/400 hydrochloric acid for measurements of activity and protein content. At the same time samples of 3 ml were taken for crystallization. These were mixed in test tubes with 2 ml of saturated ammonium sulfate and the pH was adjusted to 5.6 by means of several drops of 1 N sulfuric acid. The mixtures were then left at 20°C for crystallization. The crystallization mixtures were filtered after 24 hours and the protein content of the filtrates was determined, the percentage yield of crystallized protein was then calculated.

TABLE I

*Formation of Gamma from Chymotrypsin at 35°C*

50 gm alpha crystal cake + 75 ml water + 75 ml 0.4 M borate buffer pH 9.0, 4 hrs at 35°C

Material	Total activity	Total protein nitrogen	Specific activity
	$[TU]_{ml}^{Hb}$	mg	$[TU]_{mg}^{Hb} P.N$
Original solution	134	2960	0.045
Original solution after 4 hrs at 35°C	101	2260	0.045
Per cent	75	76	
First crop of gamma	49	1045	0.047
Mother liquor	39	875	0.045
First recrystallization of gamma	45	880	0.051
Second " " "	37	780	0.048
Third " " "	32	670	0.048
Fourth " " "	29	560	0.052

The results are given graphically in Fig. 8. It is to be seen that crystals of chymotrypsin appeared only in the samples taken within the first 2 hours, while the later samples contained crystals of gamma protein. The rate of crystallization of each species of crystals is affected by the presence of the other materials so that there was a distinct minimum in the amount of crystalline precipitate in the sample tubes, as shown by the plotted curve for the per cent of protein crystallized. If the crystallization were allowed to proceed for a longer time the difference in the yields would gradually disappear, except for the difference between the solubility of the two species of crystals. The curves for activity and protein content of the original

chymotrypsin mixtures show that the change of chymotrypsin at 35°C and pH 7.6 is accompanied by a simultaneous loss both of activity and protein which means that there is no accumulation of inert protein in the solution and hence the specific activity of the protein remains unchanged during the whole process. The yield and the specific activity of gamma on recrystallization are shown in Table I.

### 5 Final Method for Isolation of Beta and Gamma Crystals

The final procedure adopted for the isolation of the new enzymes follows.

As a starting material either chymotrypsin or the mother liquor from chymotrypsin crystallizations can be used. In the latter case the protein is first salted out in 0.7 saturated ammonium sulfate and the precipitate is then used in the following operations in the same manner as the crystal cake of chymotrypsin.

*Isolation of Gamma Crystals*—Suspend 100 gm of crystal cake of chymotrypsin in 100 ml water. Add 50 ml 0.5 M phosphate buffer pH 8.0 and store the clear solution for 3 weeks at about 5°C. Then add 120 ml saturated ammonium sulfate, adjust the pH to about 5.6 by means of 5 N sulfuric acid, added drop by drop, and allow the mixture to stand at 20°C for crystallization of gamma chymotrypsin. Filter after 3 days with suction. The filtrate (first gamma mother liquor) is stored at 5°C. Yield about 30 gm gamma filter cake.

*Recrystallization of Gamma*—Recrystallize the gamma crystals by dissolving 10 gm in 30 ml water and adding 20 ml saturated ammonium sulfate. Filter after 24 hours with suction. Residue—second crystals of gamma stored at 5°C.

*Isolation of Crude Beta Crystals*—Combine filtrate with the first gamma mother liquor, adjust pH to 4.2 with 5 N sulfuric acid, and salt out the protein by adding 21 gm solid ammonium sulfate to each 100 ml of solution. Filter with suction. Dissolve 10 gm of amorphous precipitate in 7.5 ml N/100 sulfuric acid and allow solution to stand for several days at 20–25°C until a heavy precipitate of fine needle crystals of crude beta is formed. The solution frequently turns completely into a thick fibrous gel of crystals. Filter with suction. The filtrate on standing may yield another crop of needle crystals. The total yield is about 50 gm crude beta filter cake per 100 gm of original chymotrypsin filter cake.

*Recrystallization of Crude Beta*—Dissolve 10 gm of crystal cake in 30 ml of water and add 30 ml saturated ammonium sulfate. Adjust pH to 5.6 by means of a few drops of 5 N sodium hydroxide, and after inoculation with gamma crystals allow the solution to stand for several days at 20°C. Filter off any gamma crystals formed and adjust the pH of the filtrate to 4.2. Crude beta crystals gradually appear. Filter after several days and repeat crystallization of the crude beta in the same manner.

*Isolation of Pure Beta Crystals*—Dissolve 10 gm of three times recrystallized crude beta crystal cake in 250 ml of water, add 10 ml 0.4 M borate buffer pH 9.0

Heat solution to 37°C and then let it stand at this temperature for 1 hour. Cool solution to 20°C and adjust pH to 4.2 by means of 5 N sulfuric acid. Add with stirring 65 gm of ammonium sulfate and if a precipitate is formed add 5 gm Standard Super-Cel and filter with suction through 9 cm No. 3 filter paper. Dissolve 21 gm ammonium sulfate in each 100 ml of the clear filtrate and filter the formed amorphous precipitate of protein with suction on hardened paper. Reject filtrate. Dissolve each gram of precipitate in 3 ml water and add 2 ml saturated ammonium sulfate. Adjust solution to pH 4.2 and let it stand at 20°C. An amorphous precipitate forms which gradually changes into very fine crystals. Filter after several days with suction, yield about 3 gm of pure beta crystal cake.

The procedure for recrystallization of the "pure" beta crystals is the same as for the recrystallization of the crude beta.

## *II Physical and Chemical Properties of the Various Crystalline Proteins*

### *1 Solubility in 0.4 Saturated Ammonium Sulfate pH 4.0 at 10°C in Presence of Increasing Amounts of the Solid Phase*

*Experimental Procedure*—The following operations, except crystallization, were done in a constant temperature room at 10°C  $\pm$  0.5. The concentration of ammonium sulfate is expressed in terms of saturation at 20°C.

*Crystallization*—The crystalline proteins used in the solubility tests were recrystallized several times in 0.4 saturated ammonium sulfate made up in M/10 acetate buffer of the same pH as that used in the solubility tests.

*Washing of Crystals*—10–15 gm of crystal cake was suspended in 0.4 saturated ammonium sulfate in a 100 ml Pyrex test tube provided with a glass bead, 15 mm in diameter. The tube completely filled with the ammonium sulfate solution was stoppered with a one-hole stopper and finally plugged with a short glass rod. Care was taken not to leave any air space in the tube. The tube was then placed horizontally in a rocking machine and allowed to rock at a slow rate so as to cause the glass bead to roll back and forth all along the tube thus keeping the suspension continuously stirred. The rocking was generally continued for 24 hours, the suspension was then filtered with suction and the clear filtrate was analyzed for activity and protein concentration. The filtered crystals were then resuspended in fresh 0.4 saturated ammonium sulfate and the whole procedure was repeated several times until the filtrates gave constant values for activity and protein concentration. These values generally became constant after 2 or 3 washings.

*Solubility at 10°C in 0.4 Saturated Ammonium Sulfate*—The crystals from the final washing were uniformly suspended in 50 ml of 0.4 saturated ammonium sulfate. Increasing amounts of the uniform suspension from 0.1 to 15 ml were made up to 15 ml with 0.4 saturated ammonium sulfate and then transferred into 15 ml test tubes, each provided with an 8 mm Pyrex glass bead. The tubes were stoppered with one-hole rubber stoppers and then plugged with short glass

rods so as to remove all the air from the tubes. The suspensions were rocked for 24 hours and then filtered through small No. 42 filter papers. The clear filtrates, as well as the original suspensions, were analyzed for activity and protein concentration. Stirring of the suspensions for longer than 24 hours did not affect the solubility in either direction, thus proving that equilibrium was reached practically in 24 hours.

Fig. 9 shows the curves for the solubility at 10°C of the various crystals in 0.4 saturated ammonium sulfate pH 4.0 in the presence of increasing amounts of crystalline protein added to the system. There

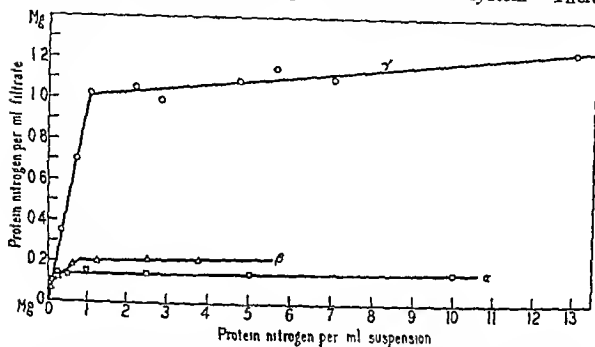


Fig. 9 Solubility of alpha, beta, and gamma chymotrypsins in 0.4 saturated ammonium sulfate pH 4.0 in the presence of increasing quantities of solid phase. Per cent impurities calculated from the slopes of the lines (3)  $\alpha = 0$ ,  $\beta = 13$ ,  $\gamma = 20$ .

is an enormous difference in the solubility of the gamma crystals as compared with those of beta or alpha trypsin crystals, the solubility of the last two proteins being about the same.

The solubility curves of alpha, as well as of beta and gamma crystals, consist of intersecting straight lines and correspond to the theoretical phase rule curves of substances mixed with an amount of impurity varying from 0 to 13 per cent as determined from the slopes of lines.

The solubility curve for the crude beta crystals, unlike the other curves, consists entirely of a continuous curvature similar to the curve

shown in Fig 3 and hence corresponds to a solubility curve typical of a solid solution

*Mixed Solubility*—Saturated solutions of the various crystals in 0.4 saturation ammonium sulfate pH 4.0 were made up by suspending 15 gm of washed crystal cake in 100 ml of solvent and shaking the suspension for 24 hours at 10°C in the manner described before. Several portions of 15 ml of the well mixed suspension were filtered with suction on separate papers. The filtrates were combined while each of the residues was stirred in a 15 ml test tube with saturated solutions of various proteins or with its own saturated solution and also with 15 ml of fresh

TABLE II

*Solubility of Alpha (Chymotrypsin), Beta, and Gamma Crystals in 0.4 Saturated Ammonium Sulfate Made Up in M/10 Acetate Buffer pH 4.0 and Saturated with the Various Crystals*

Solid phase	$\alpha$			$\beta$			$\gamma$		
	$\alpha$	$\beta$	$\gamma$	$\alpha$	$\beta$	$\gamma$	$\alpha$	$\beta$	$\gamma$
Liquid phase-saturated solutions of	$[TU]^{Hb} \times 10^{-3}$ per ml filtrate								
After 24 hrs	8.4	19	46	18	11	39	49	52	58
After 48 hrs	8.4	21	51	19		40	54	60	62
Calculated		19.4	68	19.4		71	68	71	
Mg protein nitrogen per ml filtrate									
After 24 hrs	0.134	0.33	0.87	0.33	0.19	0.80	0.99	1.05	1.23
After 48 hrs	0.132	0.35	0.93	0.34		0.76	1.05	1.20	1.20
Calculated		0.32	1.35	0.32		1.41	1.35	1.41	

0.4 saturated ammonium sulfate pH 4.0. The tubes were provided with beads. The suspensions were rocked for 3 hours, then filtered with suction. The filtrates were rejected while the crystalline residues were resuspended in corresponding fresh solutions and rocked for 24 hours and then filtered. The filtrates were analyzed for activity and protein concentration while the residues were resuspended once more in corresponding fresh solutions and rocked for 48 hours longer and then filtered. The final filtrates were analyzed for activity and protein concentration as before. The results of these determinations are given in Table II which shows that a saturated solution of crystals of alpha or of beta in dilute ammonium sulfate pH 4.0 continues to dissolve crystals of the other protein until the total concentration of protein dissolved equals the sum of the solubilities of the two proteins. On the other hand, a saturated solution of gamma crystals at pH 4.0 loses protein when mixed with an excess of crystals of either alpha or beta.

This is due to the fact that at pH 4.0 gamma protein combines with either alpha or beta to form crystals of a solid solution. The solubility of the solid solution crystals formed is apparently lower than that of the gamma crystals.

It is to be noticed that the solid solution combination between gamma and the other crystals occurs only at pH about 4.0 but not at pH above 5.5, hence it was found possible to separate the gamma protein from its combination in solid solution with either beta or alpha chymotrypsin by fractional crystallization at pH 5.5.

### *Solubility Curves of Artificial Mixtures of Crystals*

Increasing amounts of the stock suspensions of mixtures of crystals of two proteins were made up to 15 ml. with 0.4 saturated ammonium sulfate and stirred for 24-48 hours at 10°C. in the rocking machine as described before. The suspensions were then filtered and the filtrates were analyzed for protein content.

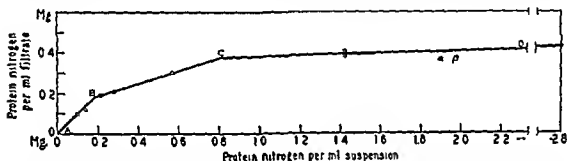


FIG. 10 Solubility curve of artificial mixture of crystals of alpha and beta chymotrypsin (65 per cent alpha + 35 per cent beta) in 0.4 saturated ammonium sulfate pH 4.0 at 10°C. in the presence of increasing quantities of solid phase. Slope of B C, measured = 0.31, calculated = 0.35.

Fig. 10 represents the solubility curve at pH 4.0 of a mixture of crystals of 65 parts of alpha and 35 parts of beta chymotrypsin. The curve corresponds to the theoretical curve of a mixture of two independent chemical substances, the solubility of either one of which is not affected by the presence of the solid phase of the other substance.

The same holds true for a mixture of crystals of alpha and gamma chymotrypsin in 0.4 saturated ammonium sulfate pH 5.5. The solubility curve shown in Fig. 11 corresponds to the theoretical curve of a mixture of two independent solid phases.<sup>1</sup>

<sup>1</sup> The samples of the enzymes used in the 'artificial mixtures' experiments contained small amounts of soluble impurities and this is shown by the fact that the solubility of the mixtures, as well as of the individual proteins, increases slightly with increase in the amount of solid phase even in the presence of a large excess of



On the other hand, the solubility curves (Figs 12 and 13) of the mixtures of gamma crystals with either alpha or beta chymotrypsin

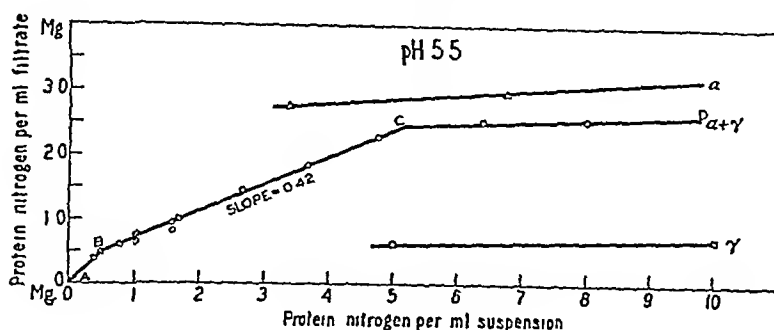


FIG 11

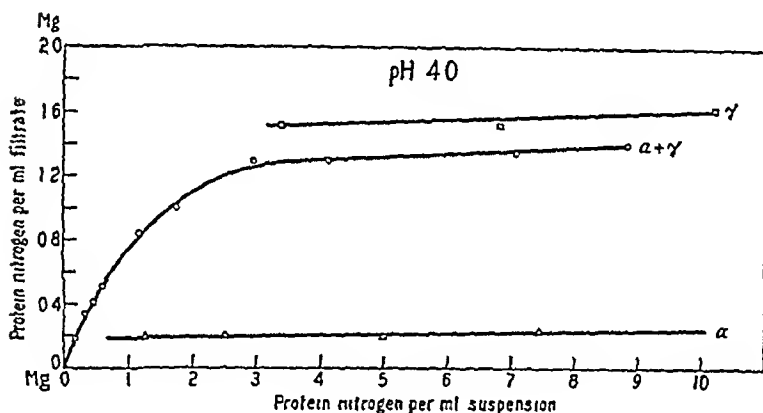


FIG 12

FIGS 11 and 12 Solubility curves of artificial mixtures of crystals of alpha and gamma chymotrypsin (40 per cent alpha + 60 per cent gamma) in 0.4 saturated ammonium sulfate of pH 4.0 and 5.5 at 10°C in the presence of increasing quantities of solid phase. At pH 4.0 the curve is of solid solution type while at pH 5.5 the curve corresponds to the theoretical curve of a mixture of two independent solid phases. Slope of B C, measured = 0.42, calculated = 0.43

crystals in 0.4 saturated ammonium sulfate pH 4.0 show clearly that gamma crystals form a solid solution with either one of the other

crystals. The data, however, are not sufficient to enable the quantity of the impurities to be determined so the exact values for the solubilities of the individual proteins used in these artificial mixtures experiments are unknown. Hence no comparison can be made between the solubility of the mixtures and the sums of the solubilities of the two components of each mixture.

proteins It is to be observed that the order of the solubility of alpha and gamma chymotrypsin crystals at pH 5.5 is reversed from that at pH 4.0, the solubility of gamma crystals at pH 4.0 being about

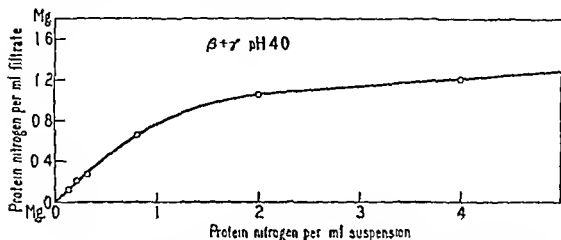


FIG 13 Solubility curve of artificial mixture of crystals of beta and gamma chymotrypsin (35 per cent beta + 65 per cent gamma) in 0.4 saturated ammonium sulfate pH 4.0 at 10°C. The curve is of solid solution type.

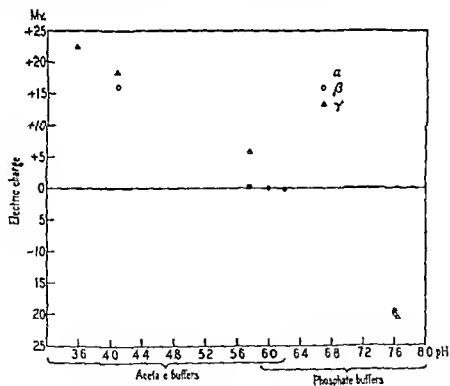


FIG 14 Cataphoresis measurements and isoelectric point determinations.

eight times as great as that of alpha chymotrypsin, while at pH 5.5 the crystals of alpha chymotrypsin are six times as soluble as the gamma crystals.

2 *Cataphoresis*—Measurements were made of the rate of cataphoretic migration of collodion particles suspended in 0.01 M buffer solutions of various pH and containing 0.06 mg of the various crystalline proteins per milliliter

The results are given in Fig 14 which shows that the isoelectric point is approximately identical for the three proteins

3 *Acid and Alkali Titration Curves*—Fig 15 shows the pH titration curves for the various proteins The data were corrected for

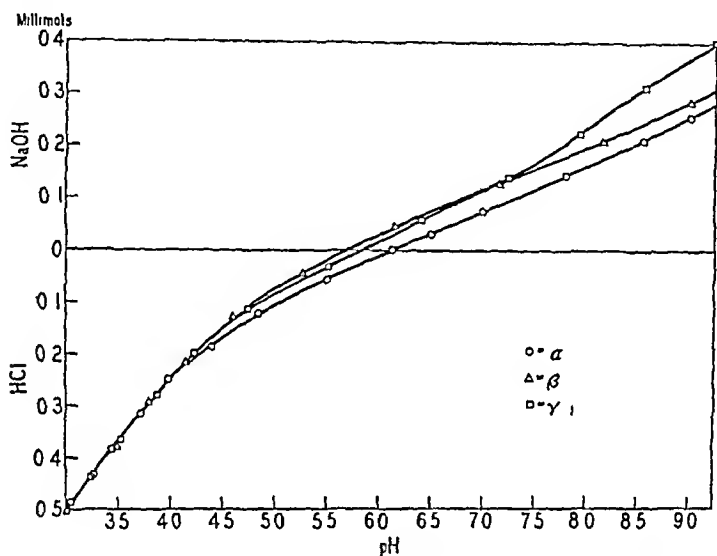


FIG 15 Acid and alkali titration curves Millimols combined per gram protein at various pH

free acid or base The curves coincide on the acid side of the isoelectric points but diverge considerably in the range of pH 5.0–9.0

*Experimental Procedure*—The various crystalline proteins were dialyzed for 18 hours at 5°C in running N/200 hydrochloric acid by the method of Kunitz and Simms (4) 10 ml samples of the dialyzed material made up in 0.1 M sodium chloride and containing a total of 3.1 gm of protein were titrated with 0.1 M acid or alkali The pH was measured by means of a low resistance glass electrode of Mouquin and Garman type (5) inserted in the titration vessel

The corrections for free base in solution were negligible in the range of pH measured The correction for free hydrochloric acid was made in the usual manner, assuming an ionic strength of  $\mu = 0.1$  as due only to the salt and acid added, hence the activity coefficient of  $\gamma_{H^+} = 0.84$  was used (6)

The isoelectric points were obtained from Fig 14

4 *Denaturation in 0.1 N Hydrochloric Acid at 25°C*—The three proteins when made up in 0.1 N hydrochloric acid rapidly undergo denaturation which is demonstrable by the formation of protein insoluble in 1.0 M sodium chloride. Native protein is soluble in this concentration of sodium chloride.

*Experimental Procedure*—10 ml of a dialyzed solution of each material containing 1 mg protein nitrogen per ml was mixed with 10 ml of 0.2 N hydrochloric acid and left at 25°C.

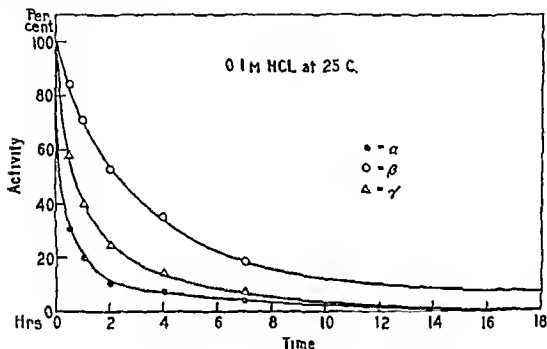


FIG. 16 Stability in 0.1 N hydrochloric acid at 25°C

Samples of 2 ml were taken at various times and neutralized with 2 ml N/10 sodium hydroxide. 1.0 ml of the neutralized solution was further diluted with N/400 hydrochloric acid to the proper concentrations for activity and protein nitrogen analysis while 2.0 ml of the neutralized solution was added to 2 ml 2 M sodium chloride made up in N/200 hydrochloric acid. The precipitate of denatured protein was filtered after 24 hours and the filtrate was analyzed for protein content. The amount of denatured protein was then found by difference.

The results are given in Fig. 16 which shows that alpha chymotrypsin is denatured in acid solution much faster than either beta or gamma.

5 *Inactivation at pH 9.0 and 35°C*—The three enzymes when brought to pH 9.0 and kept at 35°C undergo a gradual autolysis with a simultaneous loss of activity, but the rate of autolysis is more

rapid for beta or gamma than for alpha chymotrypsin as shown in Fig 17

A comparison of these results with those given in Fig 16 shows that in acid solution alpha is less stable than beta or gamma, while at pH 9.0 the order is reversed and beta and gamma are less stable than alpha chymotrypsin

In the range of pH 2.0-8.0 the stability of the three enzymes is about the same except for the gradual change of chymotrypsin into the new enzymes at pH above 4.0

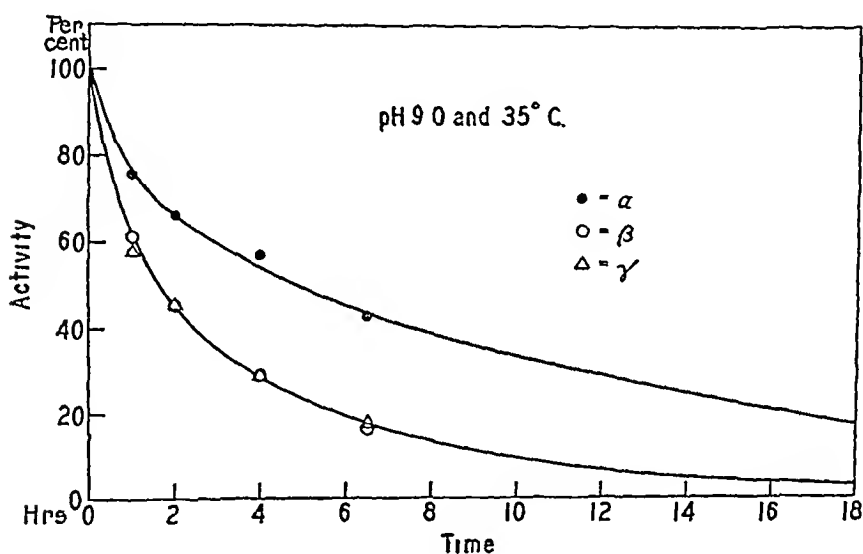


FIG 17 Stability at pH 9.0 and 35°C

6 *Inactivation by Urea* —The three enzymes partially lose their ability to digest proteins if exposed to the action of a concentrated urea solution. The effect of urea is almost instantaneous and is irreversible on dilution with water, the percentage loss of enzymatic activity increases with the concentration of urea used. The three enzymes, however, differ among themselves with respect to their stability in concentrated urea solutions as shown in Fig 18, alpha being the most and gamma the least stable of the three enzymes.

7 *Denaturation by Heat* —No significant difference has been found in the stability of the three enzymes when kept at 100°C the rate of irreversible denaturation being the same for the three materials.

8 *Chemical Composition, etc*—Table III contains the data for the elementary analysis as well as for some other chemical determinations. There is generally very little difference between the three enzymes in respect to most of the chemical tests. There is, however, a striking difference in the molecular weight of the three enzymes, the molecular weight of heta being 75 per cent and of gamma only 68 per cent of that of alpha.

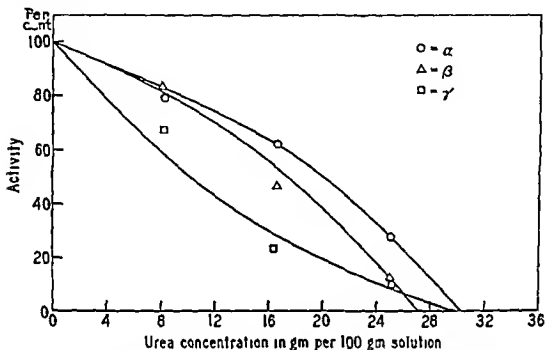


FIG 18 Inactivation by urea. 1.0 ml enzyme, 1.0 mg protein nitrogen per ml + 5 ml of various concentrations of urea in water 10 minutes at 20 C, then diluted 50 times with water for activity measurement in hemoglobin.

### III The Enzymatic Properties of the Various Chymotrypsin Proteins

The enzymatic behavior of the new crystalline proteins as well as of the original chymotrypsin towards various substrates is given in Table IV.

In practically all cases the rate of digestion by the three enzymes as measured by various methods at various pH is alike. Also the extent of ultimate digestion of casein or gelatin, as measured by the amount of free amino nitrogen formed, is identical for all three enzymes.

The three enzymes not only digest proteins with the same rate but

TABLE III  
*Physicochemical Properties*

	$\alpha$	$\beta$	$\gamma$
Elementary analysis in per cent dry weight (a)	C	49 80	50 00
	H	7 32	7 38
	N	15 83	15 81
	S	0 24	0 27
	P	0 00	0 00
	Ash	0 21	0 00
Protein nitrogen as per cent total nitrogen (b)	98	99	98
Amino nitrogen as per cent total nitrogen (c)	5 0	5 3	5 5
Tyrosine + tryptophane in milliequivalents per mg total nitrogen (d)	2 46	2 53	2 54
Optical rotation (e), $[\alpha]_{25}^D$ per mg nitrogen	-0 42	-0 41	-0 42
Molecular weight (f), by osmotic pressure, average of six determinations	40,000	30,000	27,000
	$\pm 1,000$	$\pm 2,000$	$\pm 1,000$
Protein tests	Ninhydrin (g)	+	+
	Biuret	+	+
	Millon	+	+
	Xanthoproteic	+	+

(a) The elementary analyses were carried out by Dr A Elek in Dr P A. Levene's laboratory

(b) Total and protein nitrogen in dialyzed solutions determined by micro-Kjeldahl method (7)

(c) Amino nitrogen in samples of about 2 mg total nitrogen measured by Van Slyke's manometric method (8)

(d) 10 ml containing 0.5 mg total nitrogen + 10 ml M/1 hydrochloric acid + 30 ml water + 10 ml 0.5 N sodium hydroxide + 30 ml 1/3 dilution of phenol reagent of Folin and Ciocalteu (9) Color read after 10 minutes against a similar mixture containing  $1 \times 10^{-3}$  millimol tyrosine

(e) Solution containing about 15 mg total nitrogen per ml (98-99 per cent protein nitrogen) was dialyzed for 24 hrs against running M/200 hydrochloric acid at 5°C Protein, total nitrogen, and optical rotation measured after dialysis

(f) Osmotic pressure measurement by the method of Northrop and Kunitz (10) Inside of collodion bags about 15 ml enzyme solution containing about 60 mg protein per ml made up in 1.0 M or 0.5 M ammonium sulfate Outside same concentration of ammonium sulfate as inside but without protein Triplicates were run for each salt concentration Rocked for 48 hrs at 5°C Equilibrium was reached within 24 hrs No effect of concentration of salt on the osmotic pressure was observed, proving that the Donnan effect was entirely eliminated Protein concentration measured at the end of experiment

(g) Ninhydrin test 10 ml enzyme containing 10 mg nitrogen per ml + 10 ml 24 per cent pyridine in water + 0.2 ml 2 per cent ninhydrin (triketohydrindenhydrate) in water Left at 25°C for 24 hrs, then made up to 25 ml with water No significant difference between the three materials was found in this test as well as in the other protein tests

they also attack the substrate molecule in the same place as evidenced by the fact that when the digestion has reached its final stage by the action of any one of the enzymes it cannot be extended further by the addition of any of the other enzymes

#### *IV The Mechanism of Formation of the New Enzymes*

The process of formation of beta and gamma from chymotrypsin at 5°C is accompanied by a loss of about 15 per cent of total protein and a simultaneous loss of about 40 per cent of activity, the last is due to the formation of inert protein. The transformation of chymotrypsin into gamma at 35°C is accompanied by a loss of about 35 per cent of protein as well as by a loss of an equal portion of activity. The loss of protein as well as of activity at both temperatures proceeds at a rate approximately proportional to the rate of change of chymotrypsin into the new enzymes and the rate of loss becomes negligible after the change has been completed.

The new enzymes have lower molecular weights than the original chymotrypsin. In the case of gamma the percentage difference in the molecular weight corresponds almost quantitatively to the percentage loss in total protein during its formation from chymotrypsin at 35°C. It is thus evident that the formation of the new enzymes from chymotrypsin is essentially a hydrolytic process resulting in the cleavage of the chymotrypsin molecule. The nature of the products formed is determined by the conditions of pH and temperature. The cleavage, however, is not profound enough to affect qualitatively the enzymatic nor most of the protein properties of the molecule. The number of peptide linkages split is very small so that the observed differences either in the number of free amino groups or in the number of free carboxyl groups of the various materials are small and almost within the limits of experimental error.

The activity of beta and gamma when expressed in enzyme units per milligram protein nitrogen, or per milligram dry weight, does not differ much from that of the original chymotrypsin. The enzymatic activity thus decreases in proportion to the size of the molecule, assuming that both the molecular weight and activity measurements are certain.

The slow formation at 5°C of beta and gamma as well as of inert



TABLE IV  
*Enzymatic Properties*

	$\alpha$	$\beta$	$\gamma$	Crystalline trypsin
Substrates				
Urea hemoglobin (a) in $[T U]_{\text{mg P N}}^{\text{Hb}}$	0 04-0 05	0 035-0 045	0 040-0 045	0 17
Clotting of milk (b), rennet units/mg P N	7-8	7	7-8	0
Casein in $[T U]_{\text{mg P N}}^{\text{Cas}}$ Non P N formation (c)	0 80	0 80	0 88	2 4
Casein in $[T U]_{\text{mg P N}}^{\text{Cas}}$ Formol titration (d)	0 08	0 087	0 087	0 18
Viscosity of gelatin pH 4 0 made up in 0 5 M ammonium sulfate				
$[T U]_{\text{mg P N}}^{\text{Gel viscosity}}$	7 4	7 5	7 5	100
Edestin (e) $[T U]_{\text{mg P N}}^{\text{Ed}} \times 10^{-3}$	3 8	4 5	4 4	7 0
Native crystalline egg albumin (f) $[T U]_{\text{mg P N}}^{\text{Alb S}}$	0 001	0 001	0 001	0
Carbobenzoxymethyl-L-tyrosylglycine amide (g) at 35°C Unimolecular velocity constant per hour at 35°C (digestion mixture containing 2 5 mg substrate and 0 025 mg enzyme protein per ml)	0 38	0 32	0 32	
Benzoyl-L-tyrosyl-glycineamide (h) Unimolecular velocity per hour at 5°C (digestion mixture containing 2 mg substrate and 0 006 mg enzyme per ml)	0 044	0 044	0 044	0
Other substrates Sturn Clupen Native hemoglobin	No difference in the rate of digestion by $\alpha$ , $\beta$ , or $\gamma$ was noticed			

(a) Method of Anson and Mirsky (11) 1 ml enzyme containing 0 01 mg protein nitrogen per ml + 5 ml hemoglobin Digested 10 minutes at 35°C

(b) Method of Kunitz (12)

(c) 10 ml 5 per cent casein pH 7 6 + 2 ml enzyme, 0 025 mg protein nitrogen per ml Digested at 35°C Samples of 2 ml + 2 ml 10 per cent trichloroacetic acid Heated 10 minutes at 85°C Filtered after 30 minutes Micro-Kjeldahl on 2 ml filtrate For units see (13)

protein appears to be a simultaneous process, while at 35°C and pH 8.0 the hydrolysis is rapid and only gamma protein is formed. Exposure of pure beta protein to 35°C at pH 8.0 does not change it perceptibly into gamma protein which shows that beta is not an intermediate product in the process of transformation of chymotrypsin into gamma protein. On the other hand the solubility curve of beta crystals shows that even the highly purified material still contains from 10-15 per cent of one or more impurities which cannot be removed by recrystallization. It is thus possible that beta undergoes

(d) Procedure same as (c) except that 2 ml samples were used for formol titration by the method of Northrop (14)

(e) 1 ml enzyme 0.02 mg protein nitrogen per ml + 5 ml 5 per cent edestin (Hoffmann La Roche) in 2 M sodium chloride made up in 0.1 M phosphate buffer pH 7.6. Digested 10 minutes at 35°C., then mixed with 10 ml 5 per cent trichloroacetic acid, heated 10 minutes at 85°C filtered after 30 minutes. 5 ml filtrate + 10 ml 0.5 M sodium hydroxide + 3 ml 1/3 phenol reagent. Color compared with standard tyrosine solution  $[T.U.]^{Ed} = \text{millimoles tyrosine equivalents formed per 6 ml digestion mixture per minute at 35°C}$

(f) Freshly prepared crystalline egg albumin by the method of La Rosa (15). Recrystallized twice. 1 ml enzyme 0.02 mg protein nitrogen per ml + 5 ml 2 per cent egg albumin in M/10 phosphate buffer pH 7.6. Left at 35°C for 18 hrs (+ toluene). The amount of non protein nitrogen formed was then determined as in (c)  $[T.U.]_{mg P N}^{Alb S} = \text{milliequivalents non protein nitrogen formed per minute per milligram protein nitrogen enzyme per 6 ml digestion mixture}$ . Evidently native egg albumin is slightly digested by the chymotrypsin but not by crystalline trypsin in agreement with the results reported by Balls and Lineweaver (16). The rate of digestion of egg albumin by the various chymotrypsins is about 1/800 of that of digestion of denatured casein.

(g) Artificial substrate specific for chymotrypsin synthesized by Drs Bergmann and Fruton (17) who supplied us with several of their preparations. Procedure: 5 ml substrate, 3 mg per ml in M/15 phosphate buffer pH 7.6 + 1 ml enzyme 0.025 mg protein nitrogen per ml at 35°C. Amino nitrogen in samples of 1 ml measured by Van Slyke's manometric method. The velocity constant,  $K = \frac{2.3}{t} \log \frac{A}{A - A_{\infty}}$  was found to decrease slightly with time of digestion. It was extrapolated to zero time and recorded here as such.

(h) Kindly supplied by Drs Bergmann and Fruton (17). Procedure: 25 ml substrate 2 mg per ml in M/15 phosphate pH 7.6 + 1 ml enzyme 0.025 mg protein nitrogen per ml at 5°C. Sample of 5 ml + 1 ml formaldehyde for formol titration with N/50 sodium hydroxide.

a further transformation either into gamma, which is very stable, or some other product

The writer was assisted in this work by Margaret R. McDonald and Vivian Kaufman

#### SUMMARY

A solution of chymotrypsin on slight hydrolysis undergoes an irreversible change into new proteins, two of which are enzymes and have been isolated in crystalline form. The new crystalline enzymes, called beta and gamma chymotrypsins, differ from the original chymotrypsin as well as from each other in many physical and chemical respects, such as molecular weight, crystalline form, solubility, and combining capacity with acid. The new enzymes still possess the same enzymatic properties as chymotrypsin. It thus appears that the irreversible change from chymotrypsin to the new enzymes does not affect the structure responsible for the enzymatic activity of the molecule.

The solubility curves of the new enzymes agree approximately with the curves for a solid phase of one component and furnish very good evidence that the preparations represent distinct substances. The various enzymes when mixed at the proper pH have a tendency to form mixed crystals of the solid solution type. Thus at pH 4.0 gamma chymotrypsin combines to form solid solution crystals with either alpha or beta chymotrypsin. Hence at this pH separation of gamma from either alpha or beta by means of fractional crystallization is impossible. At pH 5.0–6.0, however, each material crystallizes in its own characteristic form and at its own rate, thus a fractional separation of the various enzymes from each other becomes feasible.

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# DEVELOPMENT OF EYE COLORS IN *DROSOPHILA* SOME PROPERTIES OF THE HORMONES CONCERNED\*

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Diffusible substances showing hormone like action in modifying the development of eye color are known in *Drosophila* and in certain other insects. For details of this work and references to the literature see Kuhn (1937), Ephrussi (1938), and Beadle, Anderson, and Maxwell (1937). These substances are active when injected into, or fed to, appropriate test larvae. The so called  $v^+$  substance causes a modification of the eye color of vermilion brown ( $v\ bw$ ) animals from a pale pink towards brown. Similarly,  $cn^+$  substance induces a modification of cinnabar brown ( $cn\ bw$ ) eye color from colorless to wards brown.

Chemical investigations of the eye color hormones have been carried out by Khouvine and Ephrussi (1937), Thumann and Beadle (1937), and Becker (1937). Similar investigations have been continued with the aim of eventually isolating and identifying these hormones. This paper reports some of the further information which has been obtained primarily in regard to the properties, chemical nature, and possible methods of purification of the  $v^+$  hormone.

## *Source of the Hormone and Methods of Testing*

The  $v^+$  hormone used for this work was obtained from 24-48 hour old wild type pupae. The pupae were washed and dried at 100°C for 1 hour. This served both to inactivate the enzymes and to dry the material. The dried pupae were kept under reduced pressure until needed. The hormone was extracted from finely ground dried pupae by procedures to be described later in this paper. Small aliquots of the extracts to be tested were dried under reduced pressure at 100°C,

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weighed, and dissolved in Ringer's solution and, if necessary, the pH adjusted to 6.0. In most cases the concentration of extract in the test solutions did not exceed 0.7 per cent. The solutions prepared in this way were sealed in small glass tubes and immersed in boiling water for 10 minutes. This was found to be necessary to prevent bacterial growth and consequent toxicity for the test animals. Solutions prepared in this way retained their activity indefinitely.

The activity of the various solutions was determined by injecting them into the body cavities of *v bw* larvae shortly before puparium formation. The effect manifested itself in a modification of the eye color from nearly colorless towards brown. Ten to fifteen larvae were injected with each solution to be tested.

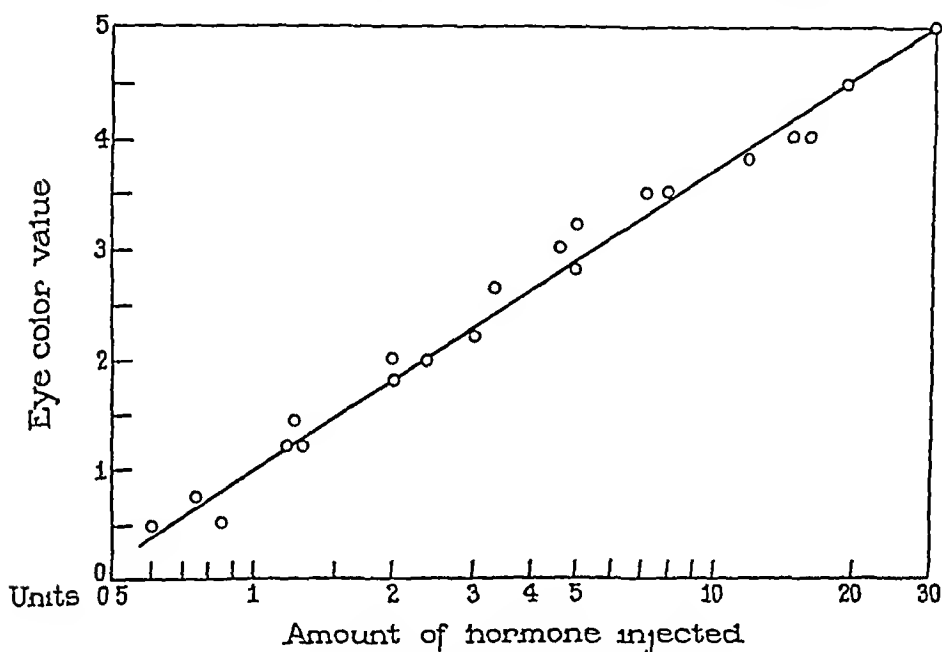


FIG. 1. Effect of concentration of  $v^+$  hormone on eye color of *v bw* flies.

**Quantitative Determination of  $v^+$  Hormone**—The development of a reasonably accurate method of determining the amount of the hormone in a given extract was necessary for exact chemical work. Dilutions of active extracts were injected into the test larvae, and the intensity of the induced eye color was graded in terms of the arbitrary color values introduced by Thimann and Beadle. The numerical values on this scale range from 0 (no response) to 5 (maximum response indistinguishable from brown control flies). In this and subsequent determinations approximately 1.4 mm<sup>3</sup> (the maximum volume practicable) was injected into each

a, and the maximum effect in each series of injections was taken as the best of the active, that solution. The results of several such dilution shown in Figure 1. The abscissa represents the amounts injected,

plotted on a logarithmic scale and the ordinates the arbitrary color values from 0 to 5. It is evident that, when plotted in this way, the relation is approximately linear. To facilitate calculations an arbitrary unit of activity was selected and defined as that amount of the hormone which when injected into one larva brings about a modification of eye color to a value of 1.0 on the color scale. In order to facilitate grading of color values and to ensure reproducible results certain genetic types of flies with different but constant eye color values were selected to form a set of standards. All test animals were compared and graded by comparison with the standard flies. The genetic composition and the eye color values of

TABLE I  
*Genetic Constitution and Color Values of Flies Used As Standards*

Constitution	Color value
Vermilion brown ( <i>v bw</i> )*	0.0
Suppressor 1 of vermillion, vermillion/vermillion brown ( <i>su<sup>1</sup>-v v bw</i> ) ♀	0.5
Suppressor 2 of vermillion vermillion brown ( <i>su<sup>2</sup>-v v bw</i> ) ♂	1.0
Suppressor 2 of vermillion vermillion brown ( <i>su<sup>2</sup>-v v bw</i> ) ♀	2.0
Brown claret ( <i>bw ca</i> )† ♀ or ♂	2.0
Eosin ( <i>w<sup>e</sup></i> )‡ ♂	3.0
Suppressor 1 of vermillion, vermillion brown ( <i>su<sup>1</sup>-v v bw</i> ) ♂	3.5
Eosin ( <i>w<sup>e</sup></i> ) ♀	4.7
Brown ( <i>bw</i> ) ♀ or ♂	5.0

\* Females of the constitution vermillion brown are somewhat darker than males. Because of this, it is desirable in critical tests to compare flies of the same sex.

† Flies of this combination have an eye color of an intensity corresponding to the color value 2, but the shade of color is not quite that desired; this standard used in most of the work reported in this paper, probably will be replaced in future work.

‡ To a lesser extent, the same objection applies to this standard as to the brown claret combination—see footnote above.

these standards are shown in Table I. The age of these standards must be controlled as the color varies slightly with age. To ensure the greatest accuracy in determination most solutions were diluted so that a color between 1.0 and 2.0 was induced.

*Extraction of the Hormone*—It has previously been shown (Khouvine and Ephrussi, 1937, and Thumann and Beadle, 1937) that the eye color hormones may be extracted from pupae with water or with alcohol ether mixtures. Various procedures were tried for the extraction of the *v<sup>+</sup>* hormone from dried wild type pupae and the following method was found most successful. The ground dried pupae were extracted several times with boiling chloroform and this inactive



extract was discarded. The hormone was then extracted by repeated treatments with hot 95 per cent ethyl alcohol. The alcohol was removed from the yellow extract under reduced pressure and the residue taken up in distilled water. The water-insoluble portion of this mixture was removed by centrifuging. The final product was a fairly clear yellow solution. The results of a number of extractions are summarized in Table II. Approximately 5 per cent of the original weight of the pupae were contained in the final extract. Disregarding a few inexplicable cases in which extracts of quite low activity were obtained, the average activity was about 280,000 units per gram extract. This means that from 1 gm. of dried pupae around 17,500 units were extracted. By using water instead of alcohol as

TABLE II  
*Extraction of  $v^+$  Hormone*

Weight of pupae <i>gm</i>	Weight of extract <i>gm</i>	Units extracted		
		Total	Per gm. extract	Per gm. pupae
0.13	0.016	700	43,700	5,380
4.42	0.101	10,500	104,000	2,380
21.5	1.28	266,000	208,000	12,360
50.0	1.32	420,000	318,000	8,400
120.0	5.30	1,484,000	280,000	12,370
160.0	8.05	3,283,000	408,300	20,520
225.0	13.30	2,240,000	168,000	9,970
110.0	5.00	2,100,000	420,000	19,100
110.0	3.86	2,100,000	544,000	19,100
50.0	1.92	1,006,600	524,000	20,130
0.5*	0.096	7,000	72,900	14,000

\* Exhaustive extraction with water only

an extractive, about the same number of units was obtained, but the extracts were less pure.

Some of the best extractions yielded 20,000 units per gram pupae, containing about 2,500 individuals. Consequently in these cases approximately 8 units were obtained from each individual. Fig. 1 shows that 30 units are required to produce the maximum effect, so that one wild type fly must have produced at least 30 units, and perhaps much more. If this figure is accepted, at most 28 per cent of the theoretical amount was recovered from the pupae. This poor recovery might be due to incomplete extraction or to partial inactivation, but in view of the relatively constant yield, it seems probable that the concentration of the hormone in the pupae at any one time does not greatly exceed this extractable amount.

*Properties of the  $v^+$  Hormone*

With the aims of obtaining some indication of the chemical nature of the hormone and of developing a satisfactory purification procedure, some of its physical and chemical properties were investigated. Unless otherwise stated, the various treatments were carried out on extracts prepared as described above. In most cases the reagents or solvents used were removed by precipitation or distillation and the residual material diluted to a known volume with Ringer's solution. If necessary the treated material or fraction was adjusted to a pH of

TABLE III  
*Heat Stability of Hormones*

Treatment	$v^+$		$cn^+$	
	Color	Units	Color	Units
100 C for 0.5 hr	3.0	6.0	3.0	6.0
3.0	3.0	6.0	2.5	4.0
12.0	3.0	6.0	2.5	4.0
36.0	2.5	4.0	1.0	1.0
1 hr at 100 C	3.0	6.0	3.5	10.0
120	2.5	4.0	2.5	4.0
140	2.0	2.5	2.0	2.4
160	0.2	0.4	0.1	0.2

\* Calculated on assumption that relationship between concentration and eye color is same as with  $v^+$  hormone

6.0, dried under reduced pressure, and reextracted with alcohol. The alcohol was removed from the extract and the residue dissolved in Ringer's solution. The extracts were then tested for activity as previously described.

*Heat Stability*—The stability of the hormones was determined by heating whole pupae in the oven at various temperatures for different periods of time. The treated material was crushed in a few drops of Ringer's solution and fed to test larvae as described by Beadle and Law (1938). This method was sufficiently exact for comparison of the different samples. The results are given in Table III. The

hormones were found to be stable at a temperature of 100°C for 1 hour. Over much longer periods of time or at higher temperatures the activity of both hormones decreased until at 160°C practically all the activity was lost in 1 hour. In general the  $v^+$  and  $cn^+$  hormones showed similar behavior towards heat. The destruction of the hormones at higher temperatures was also substantiated by comparing the activity on injection of standard extracts prepared from pupae dried at 100°C and at 130–140°C. The latter extract contained only one-fourth as much  $v^+$  and  $cn^+$  hormones.

Whereas intact dried pupae, stored at 20–25°C, retained the  $v^+$  hormone in an active state for at least 11 days (tested by feeding), ground-up material slowly decreased in effectiveness until after 11 days it was only one-fourth as active. Such stored ground material, however, seemed toxic for the test larvae, so that the apparent loss of activity may have been due to entirely different effects, *e g*, a decreased uptake of the hormone by the larvae. This seems all the more likely because no decrease in activity of hormone extracts, either in solution or in dried form, could be observed over a 4 week period of storage at 20–25°C.

*Stability to Acid and Alkali*—Small samples of active  $v^+$  extract were treated with N and 10 N sulfuric acid and sodium hydroxide at 100°C for 30 minutes. The samples were neutralized, dried under reduced pressure, and extracted with ethyl alcohol. The alcohol was removed and the residue was dissolved in Ringer's solution and tested. A control was treated with N sulfuric acid at room temperature. The results were as follows:

Treatment	Destroyed
	<i>per cent</i>
Control	0
N—H <sub>2</sub> SO <sub>4</sub>	0
10 N—H <sub>2</sub> SO <sub>4</sub>	76
N—NaOH	80
10 N—NaOH	100

The  $v^+$  hormone was stable to N acid, largely destroyed by 10 N acid and N alkali, and completely inactivated by 10 N alkali.

*Stability to Oxidation*—Beadle and Thimann demonstrated the

necessity of destroying the pupal enzymes before extraction, and suggested that the hormones are inactivated by enzymic oxidation. They also stated that hydrogen peroxide inactivated them. Some of our results led us to suspect that the  $\pi^+$  hormone might be slowly oxidized and inactivated by the oxygen in the air. We therefore tested the effect of oxygen on active extracts. It was found that bubbling oxygen through such solutions for  $\frac{1}{2}$  hour had no inactivating effect. No loss was found at pH values of 4, 6, or 9 or at pH 6 in the presence of a little charcoal as a catalyst, or at  $100^{\circ}\text{C}$  for  $\frac{3}{4}$  hour. It seems improbable that the substance is oxidized by the oxygen in the air under ordinary conditions.

An attempt was then made to repeat the results of Beadle and Thimann involving treatment with hydrogen peroxide. A sample of extract was treated with hydrogen peroxide in a final concentration of 1 per cent. The solution was boiled for  $\frac{1}{2}$  hour. The resulting solution was still active, but since it still contained peroxide, only small amounts could be injected and no quantitative results were obtained. When the excess peroxide was decomposed with freshly precipitated iron oxide the solution was toxic.

These experiments were repeated using a more concentrated extract. After heating the extract with 1 per cent hydrogen peroxide for  $\frac{1}{2}$  hour, one sample was freed from peroxide by treatment with a few drops of fresh pupal juice as a source of catalase. After 5 minutes the mixture was heated and centrifuged. The peroxide in the other sample was removed by heating with iron oxide. The sample in which the peroxide had been decomposed with catalase showed no significant loss in activity, but the iron oxide treated sample was slightly toxic and completely inactive. It was concluded that hydrogen peroxide alone does not oxidize the  $\pi^+$  hormone, but that iron oxide may act catalytically in making this oxidation possible. It was found that iron oxide also catalyzes oxidation by molecular oxygen. At a temperature of  $100^{\circ}\text{C}$  in the presence of iron oxide and molecular oxygen, 82 per cent of the hormone activity was lost in  $\frac{3}{4}$  of an hour.

*Enzymic Inactivation*—Since the hormone had been shown to be relatively stable towards oxidation, it was thought advisable to investigate its inactivation by the enzymes present in the larvae and pupae. Juice obtained by crushing or grinding fresh pupae or larvae

and filtering, was mixed with concentrated active extract. The solutions were allowed to stand for 15–20 hours on watch glasses at 30°C, then diluted with Ringer's solution to known volumes, sealed in glass tubes, heated, centrifuged, and tested by injection. The results of several experiments are shown in Table IV. In no case using juice from crushed pupae or larvae did inactivation result. However, the juice from wild type or vermilion larvae and wild type pupae obtained by grinding the material thoroughly with finely

TABLE IV  
*Enzymic Inactivation of  $v^+$  Hormone*

Enzyme			Inactivation
Source	Preparation	Treatment	per cent
Wild type pupae	Crushed	Heated	0
" " "	"	Unheated	0
" " "	Ground	"	83
Wild type larvae	Crushed	Heated	0
" " "	"	Unheated	0
" " "	Ground	Heated	0
" " "	"	Unheated	95
<i>v bw</i> larvae	"	Heated	0
" " "	"	Unheated	89
Wild type larvae	"	Heated, N <sub>2</sub>	8
" " "	"	" , O <sub>2</sub>	0
" " "	"	Unheated, N <sub>2</sub>	8
" " "	"	" , O <sub>2</sub>	62

powdered silica, almost completely inactivated the  $v^+$  hormone. The results indicate that the inactivating enzyme is intracellular and is freed only by grinding the cells thoroughly. Tests for the  $cn^+$  hormone in some of these cases showed that it was similarly inactivated.

Another experiment was made which confirmed the hypothesis of Thimann and Beadle that the inactivation is due to enzymic oxidation. The oxygen was removed from samples of heated and unheated enzyme solutions by bubbling purified nitrogen through them. After ½ hour concentrated active extract was added and the stream of nitrogen continued for ½ hour longer. Two other samples were

similarly treated with oxygen and enzyme solutions. The results of this experiment are also given in Table IV. Only when both oxygen and active enzyme were present did inactivation result.

*Solubilities of the  $v^+$  Hormone*—The solubilities of the hormone in various organic solvents were also investigated. The results are in complete agreement with those of other workers (Khouvine and Ephrussi, 1937, Becker, 1937). The substance is soluble in water, in 95 per cent ethyl alcohol, slightly soluble in 95 per cent acetone, and quite soluble in 50 per cent acetone. However, it is insoluble in ether, chloroform, benzene, and absolute acetone under ordinary conditions. Continuous extraction of the dried material, however, showed that it could be slowly extracted with absolute acetone. An

TABLE V  
*Effect of pH on Extraction with Butyl Alcohol*

pH	Hormone extracted	
	Eye color produced	Units
5.0	0.8	560
5.5	1.8	1400
6.0	2.3	2100
6.5	1.8	1400
7.0	0.9	630

active water extract was mixed with anhydrous  $\text{CaSO}_4$  to a crumbly mass, and the mixture was allowed to set overnight. Continuous extraction of this material with hot absolute acetone for 4 hours removed about 10 per cent of the hormone present.

The solubility of the  $v^+$  hormone in ethyl alcohol suggested the possible use of butyl alcohol in its purification. Samples of active solution were shaken with equal volumes of butyl alcohol at varying pH values. The butyl alcohol layer was evaporated to dryness and dissolved in Ringer's solution. The results are given in Table V. The solubility in butyl alcohol was at a maximum at pH 6.0 but decreased sharply at higher or lower pH values. This indicates that the hormone contains both acidic and basic groups with an isoelectric point at about 6.0.

Continuous extraction with butyl alcohol confirmed this evidence

A water solution containing  $v^+$  hormone adjusted to successive pH values of 9, 3, and 6 was extracted continuously with butyl alcohol. The extractions were carried out under reduced pressure to avoid possible inactivation at higher temperatures and each extraction was continued for 4 hours. The various extracts and the final extracted residues were freed from butyl alcohol and salts and tested. The original solution contained 105,000 units. The extract at pH 9 contained 5,900 units, that at pH 3 contained 16,100 units, while 44,100 units were extracted at pH 6. The residue contained only 1,750 units. These results showed that the active substance was extracted very slowly at pH 9, slightly more rapidly at pH 3, and quite readily at pH 6.

*Precipitation Reactions*—The effect of various precipitants on the hormone extracts was investigated in the hope that some would prove of value in the purification of the active principle. It was found that treatment with lead acetate and ammonium hydroxide precipitated the substance completely. This agrees with the results of Khouvine and Ephrussi. Treatment with barium hydroxide and 4 volumes of ethyl alcohol also precipitated it completely. In this case the activity could be completely restored by decomposing the precipitate with sulfuric acid. Treatment of an alcoholic solution of the hormone with mercuric chloride in alcohol did not precipitate it, and the activity was recovered in the filtrate after removing the excess mercury with  $H_2S$ . However, Neuberg's reagent (mercuric acetate and sodium carbonate) in 70 per cent alcohol precipitated the hormone. It could be recovered from the precipitate by treatment with  $H_2S$ . This precipitant is assumed to be specific for amino acids and amino acid-like substances.

*Molecular Weight of Hormones*—Since the chemical investigations had indicated the amino acid-like nature of the  $v^+$  hormone but had given no information as to the size of the molecule, it was thought profitable to determine the approximate molecular weight. The only method applicable to such a determination of an unisolated substance which is present in small amounts is the diffusion method. A complete discussion of this method including the calculation and evaluation of results and references to the literature is given by Cohen and Bruins (1923). Bruins, Overhoff, and Wolff (1931) later used

this method to estimate the molecular weights of carotin and vitamin A. Went (1928), by using agar blocks for diffusion, modified the method for the determination of the molecular weight of auxin.

Because of its simplicity, the agar block technique was used for the determination of the molecular weight of the eye color hormones.  $1\frac{1}{2}$  per cent agar blocks  $10 \times 10 \times 2$  mm were used. Block number 1 (the bottom block in the series) was prepared by adding 1.5 per cent dried agar to a concentrated extract. The other blocks contained 1.5 per cent agar in Ringer's solution. After solidification the blocks were cut to the correct size, and three plain agar blocks piled on the extract containing block. Care was taken to prevent occurrence of air bubbles between the blocks. Diffusion was allowed to proceed at  $25^{\circ}\text{C}$ . After a definite period the blocks were separated, placed in small vials, cut up, and covered with an equal volume (0.2 cc.) of Ringer's solution. After 15 hours at  $30^{\circ}\text{C}$  the solutions were removed for testing for the  $v^{+}$  hormone. It was assumed that complete equilibrium had been attained in that time.

To obtain the most accurate possible determination of the hormone concentration in the test blocks, it was desired to dilute the solutions for testing so that all should be of the same hormone concentration. This procedure avoids any errors which might be introduced through the use of the eye color standards. It was found best to assume the molecular weight and to calculate, from the theoretical distribution for that value, the dilutions of each fraction which should give test solutions of equal hormone concentration. The dilutions were so calculated as to give a color value of 1. Slight differences in color are most easily detectable in this region on the color scale.

The results of several determinations are given in Table VI. The diffusion time and the assumed molecular weight are given for each series. The table gives, for each block, the dilution made, the eye color obtained, the theoretical and found distribution expressed in parts per 10,000, and the experimentally determined value of  $D_{10}$ . With an assumed molecular weight of 150 the concentration in the diluted samples decreased markedly from blocks 4 to 1, indicating a higher molecular weight. Similarly, the data show that the molecular weight is higher than 300. However, with the dilutions made assuming a molecular weight of 500, the eye colors produced by the four



fractions were nearly equal and well within the range of experimental error and the limit of accuracy of the method. With the  $v^+$  hormone an average value for all the experiments of  $D_{20} = 0.316$  was obtained,

TABLE VI  
*Molecular Weight of Hormones*

v <sup>+</sup> Hormone							
Molecular weight	Diffusion time	Block No	Calculated dilution for equal concentration	Eye color obtained from diluted samples	Distribution of hormone per 10,000 total		Found D <sub>20</sub> <sup>*</sup>
					Calculated	Found	
150	60	4	13	2 5	5500	6800	0 28
		3	7	1 0	3200	3000	0 34
		2	2 5	0 2	1100	210	†
		1	0	0 1(?)	220	92	0 42
300	60	4	76	1 3	6100	6700	0 28
		3	37	1 1	3000	2800	0 27
		2	9	0 8	730	490	0 30
		1	0	0 5	80	41	0 34
500	109	4	26	0 8	5516	5300	0 34
		3	15	0 9	3201	3400	†
		2	5	0 9	1063	1100	0 33
		1	0	0 8	214	200	0 30
500	109	4	26	1 0	5516	5600	0 31
		3	15	1 0	3201	3200	0 32
		2	5	0 9	1063	1000	0 32
		1	0	0 6‡	214	130	0 27
cn <sup>+</sup> Hormone							
500	109	4	13	1 0	5516	5500	0 31
		3	7 5	1 0	3201	3200	0 31
		2§	2 5	1 0	1063	1100	0 31

\* Average value of  $D_{20}$  for  $v^+$  hormone was 0.316, for  $cn^+$  hormone, 0.31

† Unavoidable experimental errors in determinations made it impossible to calculate significant values of  $D_{20}$  in these cases

‡ Minimum value because of toxicity of fraction

§ Concentration of  $cn^+$  hormone in block 1 was too low to test

and the calculated molecular weight of the  $v^+$  hormone was 490. A similar determination for the  $cn^+$  hormone gave the same result, a molecular weight of 500.

Because of the limitations of the method the values obtained are

only approximate. It can be safely assumed, however, that the molecular weights of  $\nu^+$  and  $cn^+$  hormones are of the same order, with a value between 400 and 600.

#### DISCUSSION

The investigation has shown that the  $\nu^+$  hormone is probably a single chemical entity of true hormone like activity. So far there has been no evidence that more than one substance is involved. That it may rightly be classed as a true hormone is indicated by its high activity. The most active preparation yet obtained has a decided effect on eye color in a concentration of 0.23  $\gamma$  per fly. 1 gm. of this material would be sufficient to modify the eye color of 4,200,000 flies. There is every reason to believe that the active principle makes up only a small percentage of the dry weight of this material and that it will prove to be very much more active than this figure indicates.

The known properties of the  $\nu^+$  hormone indicate that it is an amino acid like compound. Its solubility in water and in ethyl alcohol and its insolubility in other organic solvents resemble the characteristic solubilities of amino acids. The extraction with butyl alcohol furnished evidence to the same effect. The influence of pH on the rate of extraction indicates the presence of both acidic and basic groups with an isoelectric point near pH 6. This behavior is also typical of the neutral amino acids.

Further evidence of its amino acid nature is found in its precipitation with Neuheger's reagent, which is assumed to be specific for amino acids and related compounds. Finally, all active extracts so far obtained give strong positive reactions with ninhydrin (triketo bydrindenhydrate), which is one of the most sensitive tests for  $\alpha$  amino acids.

Although all the data indicate the amino acid nature of the hormone, it seems probable that it is not a simple amino acid. The substance may be destroyed or inactivated by many treatments which would not affect simple amino acids. The instability of the hormone to heat, and to treatment with acid and alkali illustrate this point. Furthermore, the high molecular weight of 400 to 600 as determined by the diffusion method also eliminates the common simple amino acids from consideration.

The available information does not justify any conclusions as to

the exact chemical nature of the hormone. However, there seem to be three distinct possibilities. It may prove to be a complex single amino acid, a peptide of several amino acids, or it may be a compound of one or more amino acids with other substances. In any case it is probably not a normal constituent of tissue, since its occurrence seems to be rather strictly limited to certain insects. Khouvine and Ephrussi were unable to demonstrate any hormone activity in nitrogenous extracts of sheep brain, while similarly prepared extracts of *Calliphora* were very active. Although yeast is generally supposed to contain most known nitrogenous substances, we have had no indication that it contains this particular amino acid-like hormone.

Mention should be made here of the fact that all the data available indicate the very close chemical relationship of the  $v^+$  and  $cn^+$  hormones. No differences in their chemical properties have been found during this investigation. This substantiates the views of Ephrussi and Beadle regarding the interrelationship of the two hormones.

#### SUMMARY

The substance inducing the production of pigment in the eyes of vermilion brown mutants of *Drosophila melanogaster* has been shown to be a relatively stable chemical entity possessing true hormone-like activity.

A simple method for obtaining hormone solutions has been developed involving extraction of dried wild type *Drosophila* pupae with ethyl alcohol and water.

A logarithmic proportionality has been found to exist between the amount of hormone and the induced eye color. This relationship provides a simple method for the quantitative determination of hormone concentration in given extracts.

Larvae and pupae of *D. melanogaster* contain an intracellular enzyme which inactivates the hormone in the presence of molecular oxygen. The hormone is not oxidized under ordinary conditions with either molecular oxygen or hydrogen peroxide.

The hormone has been found to be an amphoteric compound with both acidic and basic groups and with a molecular weight between 400 and 600.

The solubility and precipitation reactions of the hormone suggest

its amino acid like nature. However, the instability to heat, acid, and alkali, and its rather restricted occurrence indicate a rather complex specific structure.

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# BIOELECTRIC POTENTIALS IN HALICYSTIS

## VII THE EFFECTS OF LOW OXYGEN TENSION\*

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Previous work<sup>1</sup> has shown that the existing or experimentally altered gradients of inorganic ions or other substances between the environment and the vacuolar sap of impaled cells of *Halicystis* have little influence upon the large and steady potential difference measurable across the protoplasm. The rôle of the latter, with its inherent gradients and asymmetries was therefore emphasized in the earlier title of this series "protoplasmic potentials." Although it seems desirable to alter this to the more general term now used, it is particularly the "protoplasmic" aspects which will be considered in the next several papers. Since the cell's  $\epsilon$   $m$   $f$  can drive an appreciable current of 5 to 10 microamperes through a completed circuit for many days, a source of energy, eventually metabolic, is indicated. Agents affecting metabolism, such as oxygen tension, temperature, light, inhibitors, and stimulants, will therefore be discussed in the next few papers, with a view to indicating how metabolism may produce or alter the bioelectric potential.

The present paper is restricted to the effects of altered oxygen ten

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<sup>1</sup> Blinks L. R., *J. Gen. Physiol.* 1934-35, 18, 409

This refers to the steady potential maintained when the vacuole is perfused with sea water or when vacuolar sap is placed outside the cell. There are transient decreases of P.D. produced by dilution of sea water, and by potassium and other salts but the P.D. soon recovers much of its original value probably because penetration or loss of the salts concerned soon equalizes the disturbed gradient. But recent work with sulfates has produced a nearly constant alteration of P.D. due to the very low mobility and slow penetration of the sulfate ion. The presence of other slow moving anions in the protoplasm may account for the normal P.D.

sion Some of the results have been described in a preliminary note,<sup>2</sup> time curves are now given for these, as well as for experiments involving the concurrent effects of other agents affecting the potential, such as KCl, sulfates, ammonia, and current flow, applied with a view to analyzing the mechanism and locus of the changes produced by low oxygen tension

#### METHODS

The methods are essentially the same as those previously employed<sup>3</sup> Electrical measurement was by compensation with a sensitive galvanometer as null instrument, more recently records have been taken with a specially adapted Leeds and Northrup "Micromax" automatic recording potentiometer making an adjustment every 3 seconds Both of these methods involve a slight current flow during brief periods of unbalance when the potential is changing rapidly, but the results differed in no respect when records were taken with a vacuum tube electrometer This identity could be predicted from the detailed study of current flow effects in this organism<sup>4</sup> Indeed, there is usually no difference between the  $pD$  as read by compensation and that from the deflection of the galvanometer when compensation is removed, except under certain conditions of low oxygen tension which will be mentioned below In this case it was actually the inability of the cell to maintain a corresponding uncompensated deflection, that indicated increased polarizability, and was tested in the  $pC$  bridge<sup>4</sup>

For altering oxygen content of the sea water bathing the cells, gas mixtures were bubbled through closed vials of about 25 cc volume, all openings except a small vent being vaselined Tank oxygen, air, and two different commercial nitrogens were used, one (Linde) with about 2 per cent  $O_2$  content, the other (Ohio Chemical Co) with about 0.2 per cent  $O_2$  The latter two were particularly convenient, giving close to the upper and lower  $pD$  limits respectively with many cells Mixtures were made by counting the bubbles of the two gas streams emerging in separate vessels from equal sized orifices, as shown in Fig 1, and mixing in a Y connector Purification to lower  $O_2$  content, when necessary, was done by bubbling the Ohio nitrogen through an absorber ("O<sub>2</sub>sorbent," or acid chromous sulfate plus amalgamated Zn which do not absorb  $CO_2$  or produce CO, like alkaline pyrogallol) The actual  $O_2$  content of the sea water was also directly tested by Winkler titration of a sample carefully withdrawn, with a minimum of contact with air Still more convenient, as being applicable in one of the closed

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<sup>2</sup> Blinks, L. R., and Darsie, M. L., Jr., *Proc Soc Exp Biol and Med*, 1937, 35, 522

<sup>3</sup> Blinks, L. R., *J Gen Physiol*, 1933-34, 17, 109

<sup>4</sup> Blinks, L. R., *J Gen Physiol*, 1935-36, 19, 867

vials of the chain itself was the polarographic  $O_2$  method of Vitek,<sup>5</sup> this has been employed for several years in respiration studies in the Stanford department of Physiology<sup>6</sup> and it deserves wider use in biology

For altering solutions in equilibrium with low  $O$  tensions without admission of air during the change, the train of vessels shown in Fig 1 was employed The

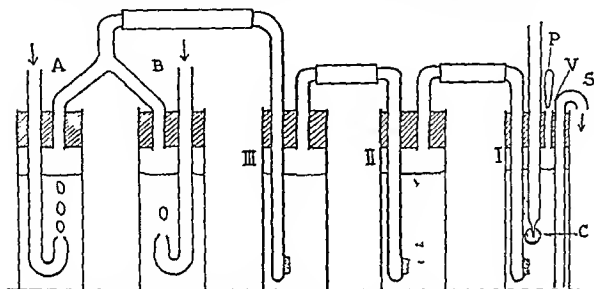


FIG 1 Apparatus for altering the oxygen content of gas mixtures, and for changing solutions in equilibrium with these mixtures, without exposing cell or solutions to air Description of its use is given in the text The glass ring support for the cell (C) and agar salt bridges making electrical contacts are omitted to avoid crowding the diagram (Shown in a preceding paper<sup>3</sup>) Vials II and III may also be used for purifying gases by absorbing  $O$ ,  $CO_2$  etc.

cell in vessel I was preceded by several other vials each with a sintered glass or fine nozzle bubbler and containing a desired solution *eg*, KCl When the

<sup>5</sup> Vitek, V Coll Czechoslovak Chem Communications 1935, 7, 537 Also *Chimie et industrie*, 1933, 29, 215

<sup>6</sup> Baumberger J P, and Muller O, reported at the winter meeting of the Western Society of Naturalists Stanford University December 1934, and the XVI International Physiological Congress Zürich 1938

A modification of this method, using a stationary electrode for following very rapid changes of oxygen tension during photosynthesis, has been described recently (Blinks L R, and Skow R K. *Proc Nat Acad Sc* 1938, 24, 420) Just after the present paper was submitted Professor Farrington Daniels informed us that he and his co workers at the University of Wisconsin had also been applying the method of Vitek for photosynthetic measurements reporting it at the Dallas meetings of the Electrochemical Society, 1938 the Photochemical Conference at Stanford University 1938 and the American Chemical Society Milwaukee, 1938 Their description is in press (*J Am Chem Soc* 1938)



P.D. had dropped in the cell to a constant level, it was assumed that the previous vessels were also in equilibrium with the gas mixture. Vessel I was then emptied by closing vent *V* with a glass plug (*P*), the pressure of the incoming gas forcing the sea water out through siphon *S*. When I was empty, vessel II was inverted and its solution was forced in through the bubbler. Sea water, meanwhile held in vessel III, was restored by similar manipulation. Where less alteration of the original sea water was necessary, e.g. where a mixture of 1 part sea water and 1 part 0.6 M KCl was desired, vial I had to be only half-filled, then no siphoning out of the first solution was needed, merely its filling from vessel II. For still smaller modifications, e.g. acidification, or addition of ammonia, small amounts of solution could be safely injected with a hypodermic syringe through the vent *V*, insufficient air being admitted to cause any change in P.D. (as tested with sea water injection). The study of current flow effects during low O<sub>2</sub> exposures presented no difficulties, all necessary connections being made in advance.

A strong agar gel in the impaling capillary helped prevent collapse of the cell during the changes of solution by the gas pressure developed in the vials.

Most of the measurements here reported have been made on *H. ovalis*, of California, but consistent results have been found in many cases with *H. Osterhoutii* in Bermuda.

The experiments were made in darkness, or in very dim light (1 foot-candle or less) too weak to affect the P.D. Greater illumination, although with negligible effect on the normal P.D. of *Halicystis*,<sup>3</sup> may become highly effective under low O<sub>2</sub> tension by restoring oxygen through photosynthesis. These and other light effects are reserved for another paper. Similarly reserved are the effects of temperature, which are again not very great upon the normal P.D. of *Halicystis*, but may markedly influence the level reached under low O<sub>2</sub> tension, or the speed with which it is attained. (Indicated in a preliminary note<sup>2</sup>) The present experiments were performed at room temperature, usually between 15 and 20°C and not varying more than 1°C in a given experiment.

For comparison with the effects of low O<sub>2</sub> on P.D., respiration measurements were made on single *Halicystis* cells in a Fenn respirometer, and on groups of four or five cells in Warburg manometer vessels. On the whole, the respiration rate fell off at about the same O<sub>2</sub> tensions as did the P.D. It was also found that oxygen consumption was markedly enhanced for some time (even for several days), after the cells were impaled, later falling off to nearly the rate found before impalement. This agrees with the greater sensitivity of the cells to lowered O<sub>2</sub> tension soon after impalement, and with other bioelectric properties at this time. Detailed report of the respiration measurements will be given in another paper.

### *General Effects of Oxygen*

It has been realized for a long time that large plant cells like *Valonia* and *Halicystis* have a very small respiration in relation to their volume, it is not inappreciable, however, amounting to 1 or 2 mm<sup>3</sup>

O per hour per cell of  $0.5 \text{ cm}^3$  volume. Considering that the immense vacuole may occupy 99 per cent of the cell's volume, this is probably as high per unit volume of protoplasm as in many marine algae. Nevertheless, the actual O consumption is so small that the cells may be kept for many days in a sealed vial of 25 cc sea water without effect upon the P.D., which remains also much the same whether the solution is stirred, aerated, or bubbled with pure  $\text{O}_2$  gas.

It is only when cells are confined in a very small volume, e.g. of 1 cc or less, that the P.D. may fall overnight due to O exhaustion. If, however, a more actively respiring organism, such as *Ulva* or yeast is also included in the vessel, the P.D. of *Halocystis* may drop very quickly on standing, to be restored by aeration (or light). Although these experiments suggest that the P.D. is sensitive to reduced  $\text{O}_2$  tension, they are not free from the possibility that  $\text{CO}_2$  or other products of the cell itself or of other organisms may be responsible for the fall of P.D., these being blown out or oxidized on aeration.

Experiments involving definite  $\text{O}_2$  content of the sea water, in equilibrium with known gas mixtures, were therefore undertaken. The first gas employed, "Linde" nitrogen containing about 2 per cent  $\text{O}_2$ , held the P.D. at practically the same value as in air as long as it was actively bubbled. When bubbling was stopped, the P.D. began to drift downward (Fig. 2). The speed with which this occurred, and the eventual level reached were not perfectly constant from cell to cell. The sex of the plant (as indicated at its previous reproductive period) may be of influence here, female plants being apparently more sensitive, and having a higher respiratory rate, possibly because of the heavier masses of unliberated gametes often remaining in the vacuole. The length of time after insertion of the capillary is also important: freshly impaled cells are more sensitive to partial lowering of  $\text{O}_2$  tension than are those which have stood for a longer time. This again is correlated with an increased respiratory rate soon after impaling (see Methods above), which would drive the  $\text{O}_2$  level down faster in the vicinity of the cell. It might be thought that the freshly bealed region in the vicinity of the capillary is more readily injured by low  $\text{O}_2$  tension than the rest of the cell surface, and so introduces a short circuit to the remaining potential source, but resistance measurements (see below) do not indicate this.

After recovery, the effects of fresh impalement may be simulated by twisting the cells upon their capillary again

As shown in Fig 2, restoration of higher  $O_2$  tension around the cell restores the P D, whether the sea water is merely agitated by stirring (with a gas-tight stirrer) or by resumption of bubbling of 2 per cent  $O_2$  in nitrogen. The fall and recovery can be repeated many

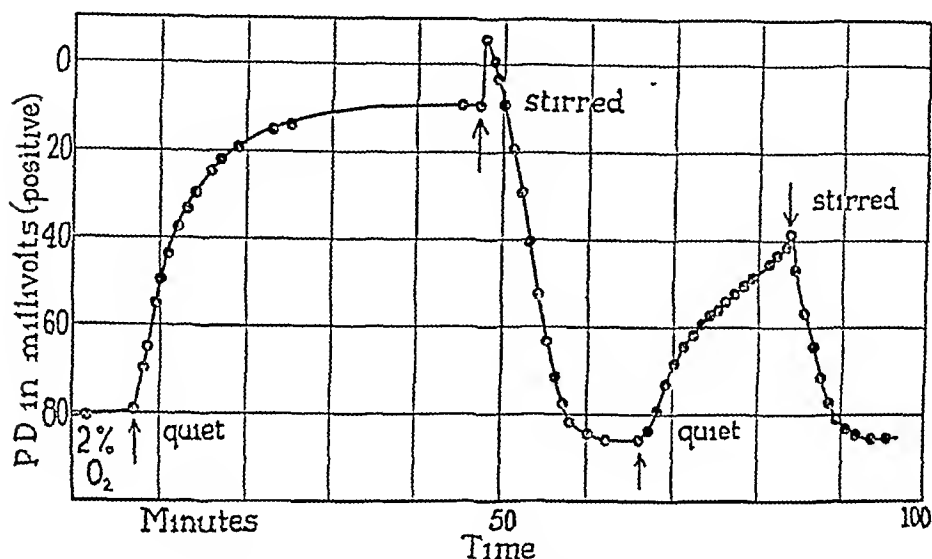


FIG 2 Graph showing the fall of potential difference across the protoplasm of an impaled cell of *Halicystis ovalis*, when bubbling of 2.0 per cent  $O_2$  in  $N_2$  is stopped (at first arrow). When the solution is quiet the cell consumes oxygen faster than it can diffuse in, and the P D drops to 10 mv positive. On stirring the sea water (with a gas-tight stirrer), the P D first falls abruptly (or even slightly reverses), then rapidly recovers. On cessation of stirring it falls again, at 40 mv stirring is resumed, producing a small downward cusp, then a rapid recovery. (Bubbling of 2 per cent  $O_2$  has the same effect as stirring.)

The ordinates are P D in millivolts, the positive sign that of the cell exterior

times with excellent reproduction of the time curves. The downward cusp at the re-introduction of higher  $O_2$ , which carries the P D lower before it restores it, is a characteristic which will be discussed later.

Since the fall of P D on cessation of bubbling does not give exact information on the  $O_2$  tension adjacent to the cell when the P D has dropped to various levels, active bubbling was continued with various mixtures of 2 per cent and 0.2 per cent  $O_2$  in nitrogen, by means of

the bubble counting mixer of Fig. 1. With a 1/1 mixture, giving about 1.1 per cent  $O_2$ , the P.D. was usually no longer maintained, but fell off to around 2/3 its value in air, as shown in Fig. 3. 0.5 per cent  $O_2$  dropped the P.D. to a still lower level, almost to that produced by 0.2 per cent  $O_2$ . And still lower  $O_2$  tensions, produced by absorbing most of the  $O$  from "Ohio" nitrogen, or allowing the cells

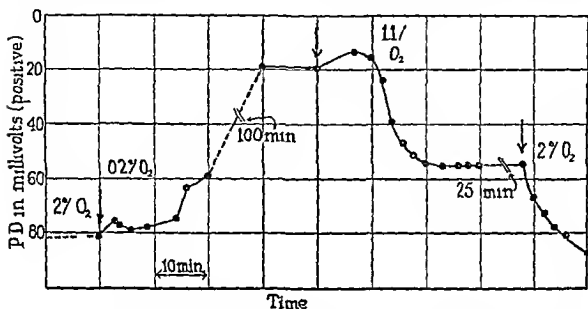


FIG. 3. Graph showing P.D. across protoplasm of impaled *H. salis* cell in sea water equilibrated for long periods with 2 per cent, 1.1 per cent and 0.2 per cent  $O_2$  in  $N_2$ . 2 per cent  $O_2$  had been bubbled for  $\frac{1}{2}$  hour previous to the beginning of the graph without reducing the P.D. appreciably below the value in aerated sea water. 0.2 per cent  $O_2$  was then bubbled, and the P.D. slowly fell during 2 hours to the constant value of 19 mv. A 1/1 mixture of the two previous gases was then bubbled (in the apparatus of Fig. 1). After the usual downward cusp, the P.D. rose to 55 mv and remained there for  $\frac{1}{2}$  hour constant bubbling. 2 per cent  $O_2$  was then restored and the P.D. promptly rose to its original level (with a slight overshooting). Intermediate mixtures of 3/1 and 1/3 of the 2 original gases giving 0.65 per cent and 1.55 per cent  $O_2$  produced intermediate levels between these P.D. values.

Ordinates are millivolts P.D. (outside of cell positive). Time intervals 10 minutes. Arrows indicate change of gas mixture.

to respire it, did not decrease this P.D. much below 5 or 10 mv, at least for periods of an hour or longer. From all these exposures, good recovery occurred when  $O_2$  tension was again raised, either to 2 per cent  $O_2$ , or to 20 per cent (air), still almost invariably with the preliminary downward cusp (Fig. 4).

It should be pointed out that equally prompt and complete recovery of P D is obtained if oxygen is restored, not in the sea water around the cell, but by perfusion<sup>1</sup> of aerated solution (sap or sea

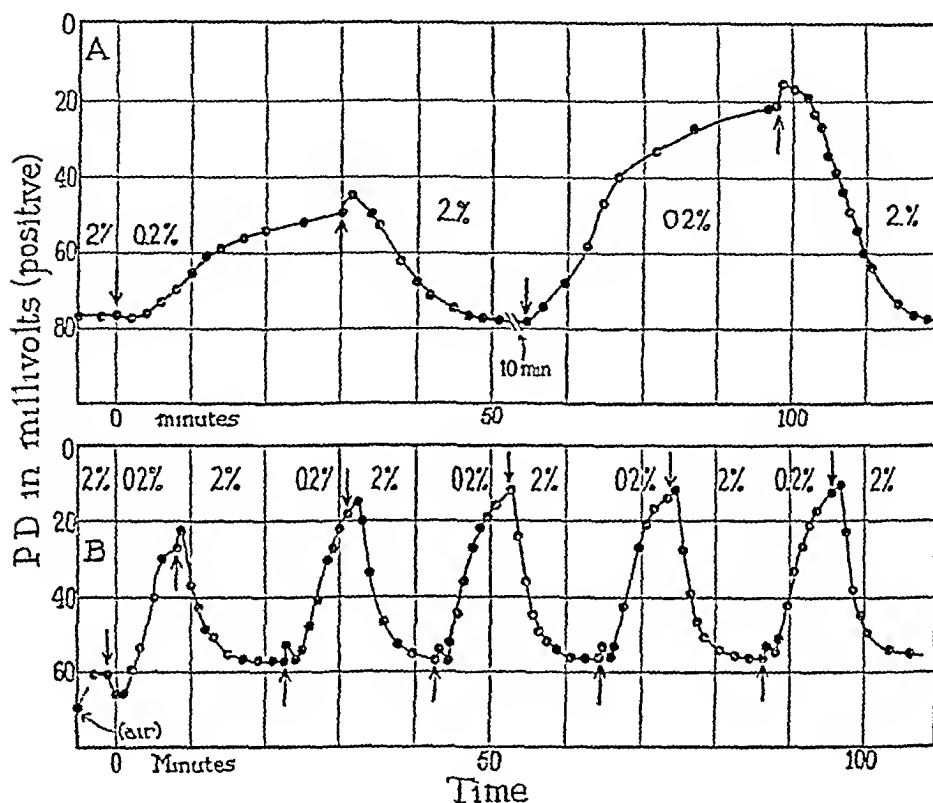


FIG. 4. Graphs showing fall of P D in unpaled cells of *H. ovalis*, when 0.2 per cent O<sub>2</sub> in N<sub>2</sub> is substituted for 2 per cent O<sub>2</sub> (each bubbling continuously). In A the fall of P D in 0.2 per cent O<sub>2</sub> is rather slow the first time, possibly because air had not been thoroughly exhausted from the vacuole, although 2 per cent O<sub>2</sub> had been bubbled for  $\frac{1}{2}$  hour previously (without reducing the P D below the a. value). After recovery in 2 per cent O<sub>2</sub>, the second fall in 0.2 per cent O<sub>2</sub> is faster and reaches a lower level. Note the downward cusps preceding recovery in each admission of 2 per cent O<sub>2</sub>.

In graph B, another more sensitive cell is shown. This had 70 mV P D in aerated sea water, which fell to 60 mV in 2 per cent O<sub>2</sub>. The fall on bubbling with 0.2 per cent O<sub>2</sub> is much faster and the P D drops to 10 mV. Readmission of 2 per cent O<sub>2</sub> promptly restores the P D. The reproducibility of the curves and the levels reached in each case are noteworthy.

Ordinates are P D in millivolts, the sign, outside of cell positive. Arrows indicate change of the gases bubbled.

water) in the vacuole (Fig 5) Thus indicates that an oxygen gradient, *as such*, is not concerned in the maintenance or restoration of the P.D., since  $O_2$  can be supplied from either side of the protoplasm. Some other gradient or structure must be responsible for the electrical asymmetry of the cells

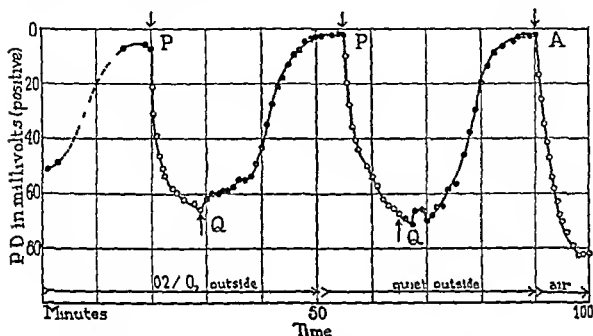


FIG 5 Graph showing course of P.D. across protoplasm of cell of *H. ovalis* impaled on a double capillary permitting perfusion of solutions in the vacuole. At the start of the graph 0.2 per cent  $O_2$  in  $N_2$  has been bubbled for 15 minutes outside the cell and the P.D. has begun to fall. This continues until the P.D. reaches about 8 mv, at which time (P) aerated sea water is perfused in the vacuole of the cell, the P.D. quickly recovers, although 0.2 per cent  $O_2$  continues to bubble outside the cell. At (Q) perfusion is stopped and the vacuolar solution remains quiet, the P.D. promptly drops again as oxygen is consumed from the vacuole. At (P) perfusion of aerated sea water is again started, but bubbling is stopped outside the cell, the P.D. rises again, although the normal oxygen gradient is now reversed. At (Q) the perfusion is again stopped and the P.D. falls. At (A) the outside sea water is aerated, and the P.D. rapidly rises to 82 mv.

The designations and horizontal arrows at the bottom of the graph refer to the state of the sea water outside the cell. Ordinates are P.D. in millivolts, outside of cell positive. Time intervals 10 minutes.

It is perhaps desirable to discuss at this point the rôle of "injury" in the fall of P.D. during exposure to low  $O_2$  tension. There is no doubt that injury eventually occurs when cells are deprived too long of oxygen. There may be visible evidence such as small holes in the

protoplasm, or its pulling away from the inserted capillary. In such cases it is easy to see why the  $P D$  may be lowered by short circuits through these visible, or invisible leaks. Usually there is no recovery when oxygen is restored to these cells, their  $P D$  soon drops close to zero (not 5 or 10 mv constantly maintained as in 0.2 per cent  $O_2$ ), and their effective resistance is very low.

It is quite otherwise with cells showing reversible depression of  $P D$  in low  $O_2$  tensions. Cells have been so kept for many hours or even overnight, showing the same low positive  $P D$  of 5 to 10 mv, and then recovering promptly when aerated. It is not likely that a partial short circuit due to injury would remain so long constant. Furthermore, as seen in a later section, the electrical resistance of such cells is *higher*, not lower, than normal. Finally, they maintain this low positive value in low  $O_2$  under conditions when they may display a *negative*  $P D$  when oxygen is restored. This could not possibly be due to a reduction of the "aerated" value by partial short circuits. This decisive behavior is seen in the next section.

### *Combined Effects of Ammonia and Low Oxygen Tension*

An earlier paper<sup>3</sup> has described the reversal of  $P D$  produced by small concentrations of  $NH_4Cl$  in the sea water. Sharp thresholds characterize both the reversal and recovery of positive  $P D$ , and the reversal may be repeated many times, or continued an hour or more at a time without apparent injury of any sort. It became of interest therefore to combine this treatment with that of low  $O_2$  tension, seeking answers to the following questions:

1 Does the presence of sub-threshold ammonia concentration in any way influence the sensitivity to low  $O_2$ , either in the effective tensions for fall of  $P D$ , the speed of fall, or the  $P D$  level reached?

2 Conversely, does low  $O_2$  tension alter the sensitivity of the cells to ammonia, *i e*, increase or decrease the threshold, or change the level of  $P D$  reached?

3 Especially, when definitely superthreshold ammonia concentrations have reversed the  $P D$ , does lowering the  $O_2$  tension merely lower the reversed, negative  $P D$  to about the same extent as it does the normal positive  $P D$ , or does it abolish the negativity altogether?

In answer to the first question, very small amounts of  $NH_4Cl$  were

added to the sea water, in concentrations from 0.0001 to 0.0005 M, which are too low to affect the P.D. appreciably. Thereafter, lowering the O<sub>2</sub> tension produced much the same effects as in untreated sea water, the P.D. falling off at the same rate, and reaching the same low levels, *except* that the ammonia appeared to exaggerate the "hump" sometimes found (Fig. 6, dotted curve) which carries the P.D. to a somewhat lower level than it finally attains, during continued bubbling of 0.2 per cent O<sub>2</sub>. Also, recovery in air tended to give a more pronounced downward or even reversed "cusp" on first admission of air, preceding the rapid recovery of P.D. The significance of these changes is seen when slightly higher ammonia concentrations are applied, and they suggest the rôle played in these anomalies of the P.D. curves by the small amounts of ammonia often found in the vacuoles of *Halicystis* cells.

At higher ammonia concentrations, close to threshold for reversal, a more complicated interplay of the two treatments occurs, which may be due to acidity. An example is shown in Fig. 6. A cell exposed to ammonia just insufficient to reverse its P.D. alone, is often found to undergo temporary reversal when 0.2 per cent O<sub>2</sub> is first bubbled, but continued bubbling brings the P.D. back to positive values, about those reached in the absence of ammonia. Reintroduction of 2 per cent O<sub>2</sub>, especially if slowly bubbled, may again produce temporary reversal (as shown in Fig. 6), but continued bubbling, at this or higher O<sub>2</sub> tensions, restores positive P.D.

This remarkable behavior is not always found, since conditions have to be adjusted so that the ammonia is close to threshold and remains near there during the bubbling (it was tested at the end of the run by Nessler reagent and found very little lowered in concentration). The suggested interpretation is as follows. In air, slightly sub threshold ammonia is unable to alter the pH of the protoplasm quite enough to produce its effects, but when respiration is decreased by lowering the oxygen supply, less CO<sub>2</sub> is produced, and the ammonia now becomes effective, with reversal of P.D. This is only temporary, however, possibly because sufficient anaerobic acidity develops to counteract the ammonia alkalinity, but more likely because later anaerobic changes inhibit the potential mechanism upon which the ammonia normally operates. The P.D. therefore



returns to the low positive value characteristic of 0.2 per cent  $O_2$ . On restoration of 2 per cent  $O_2$ , the reverse cycle occurs as the ammonia becomes again effective upon the recovering potential mechanism, or as anaerobic acidity disappears. Then as  $CO_2$  is produced in

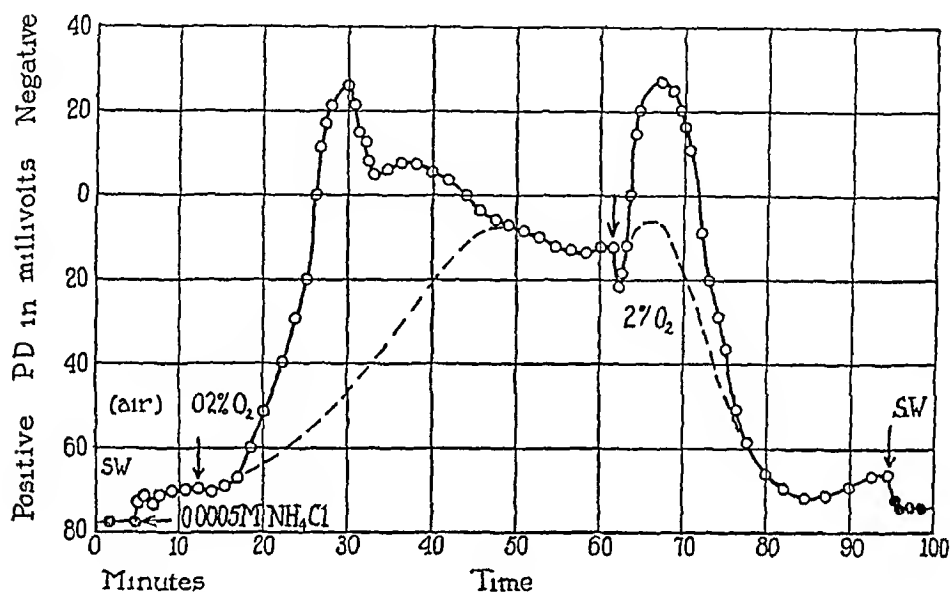


FIG. 6. Graph showing course of P D across the protoplasm of impaled cell of *H. ovalis* when 0.2 per cent  $O_2$  in  $N_2$  is bubbled in the presence of 0.0005 M  $NH_4Cl$  in the sea water (solid line). All gases including air, were bubbled through KOH previously to remove  $CO_2$  and avoid complications due to changing the pH. This low concentration of ammonia scarcely influences the normal P D (in sea water, S W), reducing it by 8 mv, to about 70 mv (positive). On bubbling with 0.2 per cent  $O_2$ , however, the P D not only begins to fall, as would be expected with low oxygen tension, but temporarily reverses in sign, becoming 25 mv negative before returning to the usual low positive value. On bubbling with 2 per cent  $O_2$ , the usual downward cusp becomes exaggerated into another temporary reversal (25 mv negative again), before recovering, with an overshooting, to its original value in air. Sea water restores normal P D.

For comparison, the dotted line indicates a characteristic time course when a very little ammonia is added to the sea water, showing residual signs of the reversal, in the "hump" during the fall, and the cusp preceding the recovery of P D. It seems possible that such residual effects are often due to the small amount of ammonia present in the vacuole and protoplasm of many cells. See text.

Ordinates are P D in millivolts, the sign being that of the cell exterior. Arrows indicate change of solution or of gases bubbling.

greater quantity, the ammonia is no longer effective, and positive  $P_D$  is regained.

Another interpretation of the effects is that ammonia is used up, possibly in the formation of proteins or other nitrogen compounds, during normal respiration, so that very low  $\text{NH}_4\text{Cl}$  concentrations are not effective in raising the internal pH of the protoplasm. When respiration is slowed up, however, such utilization of ammonia is also decreased and the internal pH is raised, causing  $P_D$  reversal. Then the potential source is affected in turn by the low oxygen tension, and the  $P_D$  falls away to its usual low positive value. When oxygen is restored, the reverse cycle is produced, the potential mechanism first recovers from anaerobic inhibition, whereupon the ammonia reversal becomes effective, then the ammonia is in turn attacked, and the  $P_D$  recovers positive values.

Whether or not either of these explanations is correct, the experimental fact is clear that oxygen tension can control the ammonia reversal, and at the threshold give rise to time courses more complex than those obtained with either ammonia or low  $\text{O}_2$  alone. The importance of interacting factors is thus demonstrated.

With slightly higher ammonia concentrations, the same tendency exists for reversal to be favored by low  $\text{O}_2$  tension, before anaerobic inhibition sets in. But *reversal*, not recovery, occurs on readmission of air, as shown in Fig. 7.  $0.001\text{ M NH}_4\text{Cl}$  was not quite sufficient to reverse the  $P_D$  alone, but bubbling of 0.2 per cent  $\text{O}_2$  caused temporary reversal, after which the usual low positive anaerobic  $P_D$  appeared. Readmission of air or of 2 per cent  $\text{O}_2$  caused the ammonia negativity to appear, without later recovery to positive aerobic values. Evidently the ammonia is now sufficient to maintain negativity, even though it was not originally able to induce it in air. Such a hysteresis has been noted before, both with altered ammonia concentrations, and with current flow assisting reversal close to the ammonia threshold. It will also be seen in the effects of temperature in another paper. Here it might be pointed out that the  $P_n$  is able to become negative again even after the slight positive  $P_n$  of low  $\text{O}_2$  tensions. This probably indicates that the anaerobic  $P_n$  is of another sort from the normal, upon which ammonia can operate.

The same inhibition of the ammonia effects is seen with higher

$\text{NH}_4\text{Cl}$  concentrations, sufficient in air to reverse the P D and maintain it negative. If reversal has already occurred during aeration, bubbling of 0.2 per cent  $\text{O}_2$  brings the P D back to the usual low

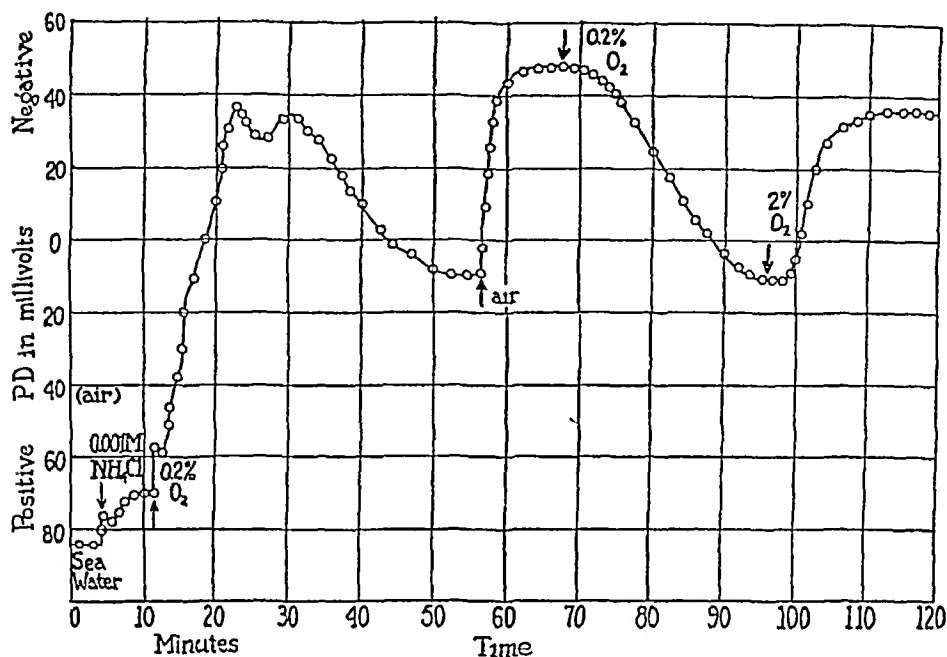


FIG 7 Graph showing course of P D across protoplasm of impaled cell of *H. ovalis* when 0.2 per cent  $\text{O}_2$  in  $\text{N}_2$  is substituted for air in the presence of 0.001 M  $\text{NH}_4\text{Cl}$  in sea water (Gases washed free of  $\text{CO}_2$  with  $\text{KOH}$  to avoid complications due to pH change). This ammonia concentration is just below the threshold for reversal with this cell, dropping the P D from 83 mv to about 70 mv (where it would stay indefinitely in air). On bubbling of 0.2 per cent  $\text{O}_2$ , the P D not only falls, as would be expected, but first undergoes a strong reversal to nearly 40 mv negative before returning to the usual low  $\text{O}_2$  level of 10 mv positive. Restoration of air then induces a strong reversal, to 50 mv negative, which is again counteracted by 0.2 per cent  $\text{O}_2$ . 2 per cent  $\text{O}_2$  is also able to induce negative P D. Only a lowering of ammonia concentration is able to abolish this negativity with higher oxygen tensions, although the negativity is only latent at 0.2 per cent  $\text{O}_2$ .

Ordinates are P D in millivolts, the sign being that of the cell exterior. Arrows indicate change of solution or of gases bubbling.

anaerobic levels, while bubbling of air again restores large negative values (much as in Fig 7). Or if the ammonia is added when the P D has been already lowered by 0.2 per cent  $\text{O}_2$ , then it has practically no effect, until oxygen is restored, whereupon the P D reverses

There is merely a "latent" negativity during low  $O_2$  exposure, which requires oxygen for its development, and becomes latent again when  $O_2$  tension is reduced

This latent negativity holds true even for rather large additions of  $NH_4Cl$ , such as would produce almost instant reversal of P.D. in air (0.01 M or higher). There is not even the initial cusp which is found at low pH values,<sup>7</sup> hence attributable to ammonium ion rather than to the penetration of undissociated base. This indicates that low  $O_2$  has not only abolished the secondary effects of ammonia but also its primary ionic mobility effects. This suggests an alteration of the ionic permeability of the surface, which will be further discussed in the next section.

If ammonia is added to the sea water when low  $O_2$  tensions have had time to carry the P.D. down only part way, e.g. from 80 to 40 mv positive, then there is usually a prompt reversal of P.D., although not to its expected negative value, after which the P.D. drifts downward and becomes again positive at its usual anaerobic level. In other words the ammonia response is lost about in proportion to the loss of positive P.D.

It should be noted that the direction of the responses to low and high oxygen tensions, in the presence of superthreshold ammonia, is the opposite of that in the absence of ammonia. The curves are nearly mirror images of those in normal sea water. *Ammonia has reversed the oxygen response.*

#### *Effects of KCl during Exposure to Low Oxygen Tension*

Increased KCl concentration was chosen as an agent which alters the potential almost certainly *via* the differential mobility of  $K^+$  and  $Cl^-$  ions in the surface of the cell. Its effect on the P.D. has been described (for both species of *Halocystis*)<sup>7</sup> as an initial sharp cusp which decreases the positive P.D. (or even temporarily reverses it slightly), after which all or nearly all of the positive P.D. is regained during 5 or 10 minutes. The recovery no doubt represents the entrance of potassium into the protoplasm, largely equalizing the initial gradient, the entrance appears to be more than simple diffusion, and prob-

<sup>7</sup> Blinks L. R., *J. Gen. Physiol.*, 1932-33 16, 147. Osterhout, W. J. V., *J. Gen. Physiol.* 1937-38 21, 631.

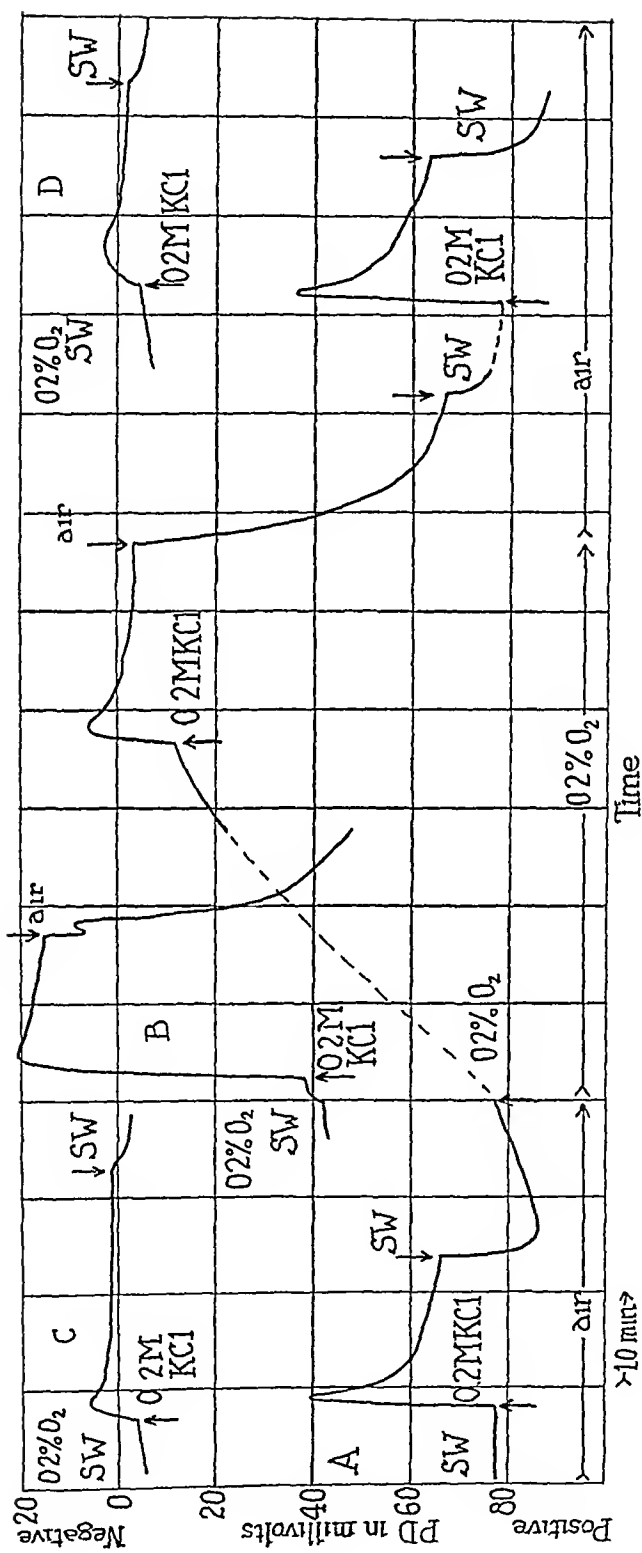


FIG 8

ably the normal accumulatory mechanism for potassium is concerned. As such, the duration of the cusp is sensitive to metabolic influences (e.g., temperature, in a way to be described elsewhere). The cusp probably reflects the high mobility of the  $K^+$  ion with respect to the  $Cl^-$  ion, and therefore is dependent on the special properties of the cell surface producing this. It was clearly desirable to study the effect of lowered  $O_2$  tension upon the cusp, and if it persisted, upon the recovery curve.

The results are shown in Fig. 8. A KCl cusp and recovery curve,

FIG. 8. Tracings of automatic potentiometer ("Micromax") records of P.D. changes in impaled cells of *H. ovalis* when sea water containing 0.2 M KCl (1 part 0.6 M KCl to 2 parts sea water) is substituted for normal sea water (S.W.) under aerobic conditions and when the P.D. has been lowered to various values by bubbling with 0.2 per cent  $O_2$ . Curve A shows a complete experiment where 0.2 M KCl is first applied in aerated sea water decreasing the original P.D. from 77 mv to 40 mv, followed by a recovery to 65 mv. Replacing sea water then produces a reverse cusp and a return to normal P.D. 0.2 per cent  $O_2$  is then bubbled through the apparatus of Fig. 1, carrying the P.D. down to about 12 mv. At this time sufficient 0.6 M KCl (also in equilibrium with 0.2 per cent  $O_2$ ) was run into vial I to bring the KCl concentration to 0.2 M. A greatly reduced cusp ensues with about half the height as with aerated KCl, and a partial recovery. When air is bubbled through the system, the P.D. recovers to about the value reached formerly with KCl in air, and readmission of aerated sea water restores it to 78 mv. After 20 minutes delay (shown by the interrupted curve), duplication of the first KCl cusp in air is then seen, indicating the cell has recovered its large response to KCl. The response is smaller if KCl is applied earlier.

If the P.D. is not allowed to drop to as low a level, through shorter bubbling or in a less sensitive cell (curve B), the KCl effect is much greater than in curve A. The cusp is also lengthened—an effect discussed in the text.

On the other hand in curves C and D where low  $O_2$  tensions have reduced the P.D. to still lower values than in A, the KCl cusp is also much smaller, and the restoration of sea water gives a smaller recovery. The apparent mobility of  $K^+$  with reference to  $Cl^-$  is lower when the P.D. drops in low  $O_2$ .

Arrows indicate change of solution or of gas bubbling, the duration of the latter being also indicated at the bottom of the graph for curve A. The fall of P.D. in the other curves has been omitted to avoid confusing the graph, but was much like those in Figs. 2, 4, etc.

Ordinates show P.D. in millivolts, the sign being that of the cell exterior. Time intervals 10 minutes. Automatic balance of the potentiometer every 3 seconds obviates the necessity of showing observed points, the lag of the instrument is usually not over 30 seconds, in following curves like these.

with the replacement of sea water afterward are first shown in air (A) Then the cell was exposed to low oxygen tension after which the KCl content was again raised to 0.2 M by means of the series vials shown in Fig 1 If this is done before the  $P D$  has fallen very low, a large response still obtains (curve B) Possibly the long duration of the cusp is due to decreased accumulation of potassium in the protoplasm, even though the  $K^+$  ion mobility is still large in the surface If the  $P D$  is first carried still lower, however, the KCl cusp is considerably reduced, as in curve A, if allowed to reach its constant anaerobic level, the cusp is greatly flattened indeed, and recovery is very slow (C and D)

This result clearly shows that the response of the cell, and probably the nature of its surface, has been altered as the result of low  $O_2$  tension, in a direction to diminish the mobility or the concentration of  $K^+$  with respect to  $Na^+$  It is shown later that  $U_{Na} = V_{Cl}$  and is not affected by low oxygen tension and since the concentration of Cl is presumably constant in the protoplasmic surface, the most probable assumption is that  $U_K$  is lowered

On re-admission of oxygen, the KCl effect also reappears (Fig 8 A), but not as rapidly as the  $P D$  itself recovers This lag both in the falling phase (Fig 8 B), and in the recovery may well indicate that  $K^+ vs Cl^-$  is not largely responsible for the normal  $P D$ , and contrasts with the sulfate effects next described

### *Effects of Sulfate vs Chloride during Low Oxygen Exposure*

It would be desirable to supplement the potassium ion effects with others, especially with those of dilute sea water which gives in *Valonia* such beautifully reproducible effects almost certainly to be referred to the much greater mobility of  $Cl^-$  than of  $Na^+$  (If  $V_{Cl} = 1.0$ , then  $U_{Na} = 0.2$ )

But in *Halicystis*, and especially in *H. ovalis*,<sup>8</sup> there is often little

<sup>8</sup>In *H. Osterhoutii*, there is often a transient reduction of  $P D$  when dilute sea water is applied, this is dependent upon the isotonic solution used as diluent, mannitol, glycerol, glucose, and other non-electrolytes giving different values (Osterhout, W J V, *Proc Nat Acad Sc*, 1938, 24, 75) They possibly alter the surface in different manners, but it would be interesting to see what the effect of low  $O_2$  would be, and it is planned to do this in the future on this species In

effect of diluting sea water with isotonic glycerol, etc., so that  $U_{Na}$  may be assumed to equal  $V_{Cl}$ . This indifference persists under low  $O_2$  tensions, so that little can be said about the changed mobilities of Na and Cl. Either they are not altered at all, or they are both reduced equally by low  $O_2$ . The latter seems more likely in view of the increased electrical resistance, and of the sulfate effects now to be described.

It was recently found that large, proportional and extremely reproducible alterations of  $P_D$  could be produced in *H. ovalis* by substitution of sulfate for chloride in an artificial sea water. The changes are almost certainly to be referred to the much greater mobility of  $Na^+$  than of  $SO_4^{--}$  in the outer protoplasmic surface (Vacuolar perfusion of sulfates has little effect). When the  $P_D$  is lowered by low  $O_2$  tension, and the sulfate substitutions made, a greatly decreased effect is obtained, indicating an approach of  $U_{Na}$  and  $V_{SO_4}$ . The resistance also rises, as shown in the next section, arguing for a decreased mobility of  $Na^+$ , rather than an increase of  $SO_4^{--}$ , in this approach.

On re-admission of air, the sulfate effect reappears *part passu* with the recovering  $P_D$  (as it also decreases in low  $O$ ). This contrasts with the more lagging KCl effect described above, and makes it seem likely that Na, and some slow moving anion like sulfate (possibly of an organic acid) may be responsible for the normal  $P_D$ .

Further details of the sulfate effect are reserved for another paper.

#### *Electrical Resistance during Exposure to Low Oxygen Tension*

Intact cells of *Halocystis* show an appreciable resistance to the flow of continuous current, although not as high as in *Valonia*, and much below that of *Nitella*. (The lower resistance may correspond with the appreciably higher NaCl content of the saps, the protoplasm allowing penetration of  $Na^+$  and  $Cl^-$  ions). The impaled cells have even less resistance, this has been shown in terms of "polarization"

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*H. ovalis* the application of very dilute sea water, or the pure isotonic non electrolytes mentioned produces a very drastic and irregular effect on the  $P_D$ , which is also believed to be due to alteration of the surface and is partly counteracted by adding calcium salts. But smaller dilutions usually have little effect.



or counter-E M F by string galvanometer records<sup>4</sup> which indicate that currents of 5 or 10 microamperes per  $\text{cm}^2$  of cell surface change the P D but slightly, and in a very slow time course (= large effective capacity)

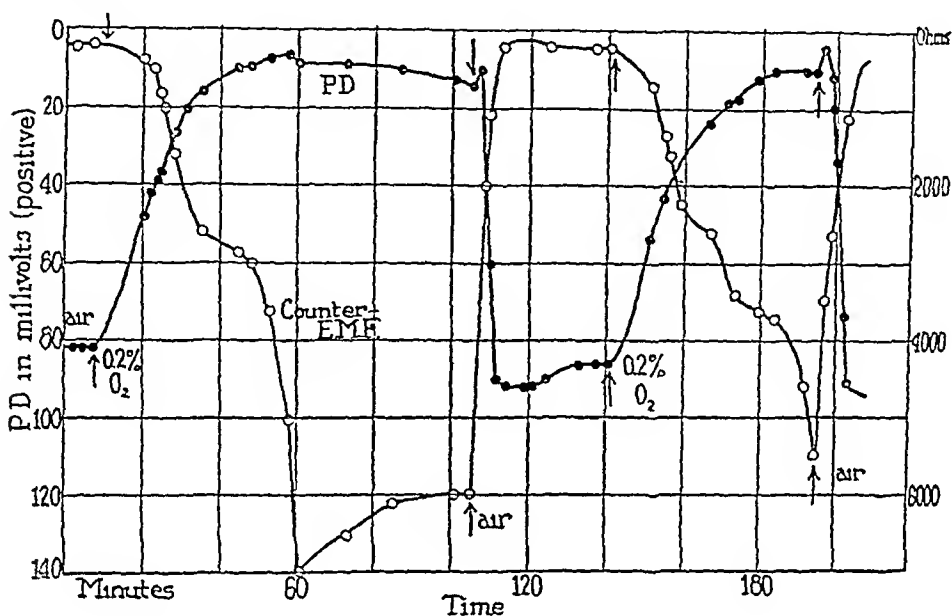


FIG 9 Diagram showing the concurrent change of P D across the protoplasm of impaled cell of *Halicystis*, and the counter-E M F developed by a flow of 20 micro-amperes passed inward across the protoplasm in a D C bridge (Ohmic resistances of capillary, etc. balanced out). The high P D is very little altered by this current flow in aerated sea water, only about 4 mv counter-E M F developing (equivalent to 200 ohms effective resistance). But when 0.2 per cent O<sub>2</sub> in N<sub>2</sub> is bubbled, the P D begins to fall, and the counter-E M F rises reaching its highest value, 140 mv (equivalent to 7,000 ohms effective resistance), when the P D has fallen to its lowest value (6 mv). It then begins to fall slightly as the P D rises.

When air is re-admitted, the counter-E M F in turn drops off as the P D recovers. The cycle is repeated on second bubbling of 0.2 per cent O<sub>2</sub>, and readmission of air.

Left-hand ordinates are P D and counter-E M F in millivolts. At the right the effective resistance, calculated from the counter-E M F, is also given. P D is black circles, counter-E M F or resistance open circles. Arrows indicate the time of admission of nitrogen or air.

It would be difficult to detect an *increased* ionic permeability under these conditions, since the effective resistance is already so

low that reductions might be insignificant, and within the limits of error. However, it was early discovered that the uncompensated P.D. of *Halocystis* was much less able to produce a steady galvanometer deflection during low  $O_2$  tensions, than when well aerated. This indicated an increased polarizability, which was directly tested in a D.C. bridge during nitrogen bubbling. It was found that the resistance rose remarkably as the P.D. fell, and eventually the cells were able to give large, prompt polarizations of 100 mv or more, as compared with a few millivolts when aerated.

The course of such a resistance change is shown in Fig. 9, plotted both in terms of counter E.M.F. developed, and in ohms effective resistance. An increase of 30 fold or more occurs during low oxygen exposure, with recovery of low resistance on aeration. It is clear that a *decreased*, rather than increased passage of ions occurs under low  $O_2$  tension. This is true both in the presence of sea water, KCl rich sea water, and sulfate sea water. An exclusion of ions would thus seem to be indicated, and could account for both the resistance rise and the P.D. fall.

The possibility is not excluded that a supply of internal ions, either organic or inorganic, did most of the current carrying during aeration, but decreased during exposure to low  $O_2$  tension. But there is no direct evidence on this point, while the altered ionic mobilities of K, Na, etc., are clear.

#### DISCUSSION

The foregoing data show beyond question that many of the electrical properties of *Halocystis* cells are dependent upon the maintenance of small, though definite oxygen tensions (e.g., 1 to 2 per cent  $O_2$  in  $N_2$ ) outside the cells. It is reasonable to presume that this involves respiration, since oxygen consumption remains constant under higher  $O_2$  tensions, but falls off when the oxygen tension falls below a certain value and the electrical properties are also affected in this region. This is hardly surprising, since the output of electrical energy is appreciable, and there must be some metabolic source for it. Furthermore, anaerobic respiration, if present, is not able to maintain more than about 20 per cent of the P.D. found in air. The important point for discussion is the mechanism by which respiration

and  $P D$  are related. At least two main possibilities suggest themselves (or with their combination, a third)

A Respiration maintains a gradient of ions, either organic (intermediate metabolites) or inorganic (possibly in connection with electrolyte accumulation) which is responsible for the  $P D$ . When oxygen is lacking, these gradients are reduced or abolished, to reappear on aeration.

B Respiration maintains the remarkable protoplasmic surfaces which are responsible for many of the unique properties of the cell. When these properties are lost or altered, the  $P D$  drops, disappears, or reverses.

C Both gradients and surfaces are altered, simultaneously or in succession.

It is easier to speculate concerning A than to adduce definite proof. Until we are able to identify some specific ionic gradient as the source of the  $P D$ , we shall not be able to say much concerning its alteration under metabolic influences. Formally, the disappearance of such active ions would account satisfactorily for both the fall of  $P D$  and the rise of effective resistance (there being fewer ionic carriers of the current which could pass the surface). However, it is difficult to see why the reduction in numbers of internal ions should so strikingly influence the effects of ions applied externally. Since there is definitely some alteration in the response of the cell toward external ions, referable to changes in the surface properties, the principle of least causes (Occam's razor) suggests that we explore this possibility fully before postulating an unknown, internal change in addition.

If surface changes be accepted as adequate to account for the altered  $P D$  and resistance, it is important to know by what mechanism these are effected under reduced oxygen tension. Here again we can only speculate, concerned as we are with the very nature of the surface, and its origin, maintenance, and alteration under metabolic influences. That it should bear such a relation is not surprising, since it is without doubt a complex lipoid in some manner built up by the cell itself, perhaps also constantly disappearing during metabolism. There is no reason to think it is a stable secretion like cellulose or chitin (although these also may have their intimate relation with the protoplasm). Its ease of formation when the cell is cut,

etc, suggest a large reserve and ready mobilization of surface materials, but what the relation of this mobilization may be to metabolism is not known. Evidently the surface is not destroyed by low  $O_2$  tension, on the contrary, the rise of resistance indicates that it becomes a more effective barrier, at least to the passage of ions. This might be due to thickening, dehydration, or lowering of the dielectric constant of the surface, or to a change in charge, number or size of "pores". In any case the cell is apparently protected against the loss of accumulated ions, or entrance of excluded ones, during temporary anaerobic conditions (cf Cowan<sup>17</sup>).

Probably the surface does not actually alter mechanically (surface tension studies would be valuable under anaerobic conditions), but some constituent is gained or lost. The hypothetical substance "R" of Osterhout,<sup>9</sup> necessary for the manifestation of high  $K^+$  mobility in *Nitella*, may be a product of respiration and tend to disappear under anaerobic conditions. Or it may be that the surface gains rather than loses something, thus the addition of guaiacol or cresol alters many of the electrical properties of *Valonia*, including both  $K^+$  mobility,<sup>10</sup> and polarizability.<sup>11</sup> While it is not suggested that a specific phenolic substance like these is produced anaerobically in *Halocystis*, it is quite likely that some metabolite may accumulate with similar effects. In general, any weak acid which can penetrate the cell tends to lower the pD, and increase the polarizability of *Halocystis*,<sup>4</sup> lactic, pyruvic or other acids known to be involved in metabolism may affect the surface similarly.

It was partly to test the possible rôle of increased anaerobic acidity that some of the ammonia experiments were performed (although the suppression of reversed pD may be merely another manifestation of reduced permeability to the ionic species involved). The increased sensitivity to ammonia during part of the anaerobic drop of potential scarcely agrees with increased acidity, although the later inhibition might be so explained. But it was found that previous exposure of the cells to weak acid (e.g. acetic) did not render them more sensitive,

<sup>9</sup> Osterhout, W J V, *J Gen Physiol*, 1933-34 17, 105

<sup>10</sup> Osterhout, W J V, *J Gen Physiol* 1936-37 20, 13. But guaiacol does not reverse the KCl effect in *Halocystis* *J Gen Physiol*, 1937-38, 21, 707

<sup>11</sup> Blinks L R. *J Gen Physiol*, 1935-36 19, 633

or quicker to lose their P D in low O<sub>2</sub> tensions. Probably the surface effects, though they resemble in some respects those produced by acidity, are due to some other anaerobic alteration. The nature of this must wait for future investigation.

It may be that some of the electrical effects of low oxygen tension correspond to the effects on permeability described with other organisms. A slightly decreased permeability to glycerol was found by Hunter<sup>12</sup> in erythrocytes under anaerobic conditions, a greater difference might be found with more polar substances. Collander and Holmstrom<sup>13</sup> describe a decreased absorption of several sulfonated dyes by plant cells under decreased oxygen tension. Decreased absorption of salts as well as of water by roots and other tissues when deprived of oxygen is well known. It is planned to test the permeability of *Halicystis* under low O<sub>2</sub>.

Whether the surface alterations here invoked can account for the bioelectric changes produced by altered O<sub>2</sub> tension in other organisms (Lund,<sup>14</sup> Francis,<sup>15</sup> Taylor,<sup>16</sup> Cowan,<sup>17</sup> Gerard,<sup>18</sup> and others<sup>19</sup>) must wait for future testing of altered ionic effects in those cases. Theories based upon oxygen effects should not overlook this possibility however.

#### SUMMARY

The potential difference across the protoplasm of impaled cells of *Halicystis* is not affected by increase of oxygen tension in equilibrium with the sea water, nor with decrease down to about 1/10 its tension.

<sup>12</sup> Hunter, F R, *J Cell and Comp Physiol*, 1936-37, 9, 15, where a few other papers are also quoted.

<sup>13</sup> Collander, R, and Holmstrom, A, *Acta Soc pro Fauna et Flora Fenn*, 1937, 60, 129.

<sup>14</sup> Lund, E J, *J Exp Zool*, 1928, 51, 291.

<sup>15</sup> Francis, W L, *J Exp Biol*, 1934, 11, 35. Francis, W L, and Pumphrey, R J, *J Exp Biol*, 1933, 10, 379.

<sup>16</sup> Taylor, A B, *J Cell and Comp Physiol*, 1935-36, 7, 1.

<sup>17</sup> Cowan, S L, *Proc Roy Soc London, Series B*, 1934, 115, 216. The increased resistance in *Halicystis* agrees with Cowan's finding that potassium was not lost from crab nerve during brief asphyxia, although the injury and action potentials were depressed. Long asphyxia, however, caused its loss.

<sup>18</sup> Gerard, R W, *Am J Physiol*, 1930, 92, 498.

<sup>19</sup> Mansfeld, G, *Arch ges Physiol*, 1910, 131, 457. Furusawa, K, *J Physiol*, 1929, 67, 325.

in the air (2 per cent  $O_2$  in N) When bubbling of 2 per cent  $O_2$  is stopped, the P.D. drifts downward, to be restored on stirring the sea water, or rebubbling the gas Bubbling 0.2 per cent  $O_2$  causes the P.D. to drop to 20 mv or less, 1.1 per cent  $O_2$  to about 50 mv Restoration of 2 per cent or higher  $O_2$  causes recovery to 70 or 80 mv often with a preliminary cusp which decreases the P.D. before it rises Perfusion of aerated sea water through the vacuole is just as effective in restoring the P.D. as external aeration, indicating that the direction of the oxygen gradient is not significant

Low  $O_2$  tension also inhibits the reversed, negative P.D. produced by adding  $NH_4Cl$  to sea water, 0.2 per cent  $O_2$  bringing this P.D. back to the same low positive values found without ammonia Restoration of 2 per cent  $O_2$  or air, restores this latent negativity At slightly below the threshold for ammonia reversal, low  $O_2$  may induce a temporary negativity when first bubbled, and a negative cusp may occur on aeration before positive P.D. is regained This may be due to a decreased consumption of ammonia, or to intermediate pH changes

The locus of the P.D. alteration was tested by applying increased KCl concentrations to the cell exterior, the large cusps produced in aerated solutions become greatly decreased when the P.D. has fallen in 0.2 per cent  $O_2$  This indicates that the originally high relative mobility or concentration of  $K^+$  ion has approached that of  $Na^+$  in the external protoplasmic surface under reduced  $O_2$  tension Results obtained with sulfate sea water indicate that  $Na^+$  mobility approaches that of  $SO_4^{--}$  in 0.2 per cent  $O_2$  P.D. measurements alone cannot tell whether this is due to an increase of the slower ion or a decrease of the faster ion A decrease of all ionic permeability is indicated, however, by a greatly increased effective resistance to direct current during low  $O_2$  Low resistance is regained on aeration

The resistance increase resembles that produced by weak acids, cresol, etc Acids or other substances produced in anaerobiosis may be responsible for the alteration Or a deficiency of some surface constituent may develop

In addition to the surface changes there may be alterations in gradients of inorganic or organic ions within the protoplasm, but there is at present no evidence on this point The surface changes are probably sufficient to account for the phenomena



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# THE INFLUENCE OF THE MOLECULAR WEIGHT OF ANTIGEN ON THE PROPORTION OF ANTIBODY TO ANTIGEN IN PRECIPITATES

## II A STATISTICAL EXAMINATION OF AVAILABLE DATA, INCLUDING SOME PREVIOUSLY UNPUBLISHED

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Massachusetts Memorial Hospitals, Boston)

(Accepted for publication October 7 1938)

In a previous publication (6) we called attention to the fact that the data then available on the ratio of antibody to antigen in neutral precipitates indicated that the ratio was importantly influenced by the molecular weight of the antigen, further, assuming the molecule of antigen to be spherical and that in neutral mixtures its surface is just about completely coated by a layer of antibody, each molecule of which is supposed to consist of a limited number of flexibly connected spheres of molecular weight equal to the "Svedberg unit" (at that time estimated to be 34,500), then it could be calculated by spherical trigonometry that the theoretical relation between ratio by weight of antibody to antigen and the molecular weight of the antigen should be approximately<sup>1</sup>

$$R = \frac{34\,500 \left[ 2 + \frac{90}{\tan^{-1} \sqrt{\tan(3\theta/2) \tan^2(\theta/2)}} \right]}{M} \quad [1]$$

where  $R$  represents the ratio by weight of antibody to antigen,  $M$ , the molecular weight of the antigen,  $\sin \theta = \rho/(1 + \rho)$ , and  $\rho = \sqrt[3]{34,500/M}$

Later (34), Svedberg revised his figure of 34,500 to 35,200, and

<sup>1</sup>This approximate formula developed by us is amply accurate for our purposes. The general mathematical problem involved was too difficult for us to solve and apparently has never been considered by any professional mathematician

though the difference is not large, we have substituted the latter figure for 34,500 in all our present calculations

To simplify computation the above somewhat unwieldy expression can be replaced, with sufficient accuracy in the experimental range, by the following empirical equation containing four arbitrary constants

$$R = 37,800 M^{-0.8} + 179 M^{-0.25} \quad [2]$$

The few data at first available (6) have now been supplemented by a relatively large amount of published material and we have examined additional systems in order to test the applicability of our theoretical relation. Here, all the data at present available are analyzed to see if the ratio is closely connected with the molecular weight of the antigen, and if so, whether our expression has a reasonably good predictive value.

Ratios for precipitates made at the constant-antibody optimum, and for precipitates made at the equivalence point (mid-point of the equivalent zone) are included. In many cases these points appear to coincide, but they may differ considerably. The theoretical relation holds much better for the "optimal" than for the "equivalent" precipitates in the case of *Vimparus* hemocyanin. Most of the published data do not afford a comparison.

### Summary of Data

The following ratios, antibody ( $ab$ ) $N$ /antigen ( $ag$ ) $N$ , are for rabbit antibody except where noted.  $R_{op}$  signifies the ratio in precipitates made at the optimum,  $R_{eq}$  the ratio in equivalent precipitates. We have calculated for each antigen the mean of all the determinations, though in some cases the difference between antisera, or the results of different experimenters, makes this of doubtful utility.

*Pneumococcal Carbohydrates*—S III  $M$  (molecular weight) assumed to be about 4000<sup>2</sup>.  $R_{eq}$  found (12), (horse-antibody) 69, 85, 54, 76, 99, 85, (13)<sup>3</sup> 69,

<sup>2</sup>The molecular weight of these carbohydrates has not been finally determined, and in any case evidently varies with the method of preparation. In our original paper we assumed the molecular weight of S III to be about 4,000. In a personal communication, Dr. Heidelberger informs us that he still considers that the true value is somewhere between 1,000 and 10,000 although the unheated viscous preparations must be many times larger.

<sup>3</sup> $R = ab/ag$  obtained by multiplying the values given ( $abN/ag$ ) by 6.25

54 69, 76 84 54, 68, 66, 66, 63, 79 63 72, 59, (rabbit antibody) (17)<sup>3</sup> 4 44, 47  
54, 46, 52, 44 33, (horse antibody) 54

SI (horse antibody) (17)<sup>3</sup> 4 34, (rabbit antibody) 18

S VIII (horse antibody) (16)<sup>3</sup> 46, 56, 42

Mean 60.1 Predicted 59.5

Ovalbumin ( $M = 40,500$  (34))

Hen-Ovalbumin— $R_s \approx R_{sp}$ , found (35)<sup>6</sup> 9.8, 9.7, 9.5 9.7, 9.5, 9.8, 9.9, 12.3,  
9.4 11.3 9.6, 9.2 10.2, 10.1 8.8 10.3, 10.4, 10.2 (1)<sup>5</sup> 10.9 10.6 14.7 12.1, 11.3,  
10.7, 13.6 11.1, 9.5, 10.2 10.3, (15)<sup>4</sup> 9.0 9.6 13.9 9.8, 9.8 11.3, (21) 10.1 9.9,  
10.1 9.9 10.5, 9.7, 9.8 10.0 9.4, 9.6 9.7, 10.0, (25) 9.3 11.0 9.2 10.6 10.6 10.4,  
10.3, 14.2 13.2, 13.5, (8) 12.7, 13.2, 13.5 12.0, 12.1, 11.9, 14.1 13.0 15.1 13.0  
13.5, 9.8 11.2 16.8 13.1 13.2 11.9 12.6 11.3 13.2, 14.3 11.4 12.8 13.1 13.8  
11.0, 13.3 14.1, 13.5, 14.0, 10.4 12.0 13.6 13.9, 14.2 11.9, 14.0 12.8 13.6 13.7,  
15.1, (3)<sup>6</sup> 14.4 10.2, 11.8, 11.5, 12.7 11.9, 12.3, 12.1, 10.3

Duck Ovalbumin—(23, 25) 10.0, 11.0, 9.7, 9.4 10.6 10.9 Mean 11.5 Pre  
dicted, 12.2

Ovalbumin Arsanilic Acid  $M = 42,500$ <sup>7</sup>  $R_{sp}$ , found (31) 3.4 Predicted,  
11.8

Iodo-Ovalbumin— $M = 43,200$ <sup>8</sup>  $R_{sp}$ <sup>9</sup>, found (31) 4.6 Predicted, 11.7,  
 $M = 43,400$ <sup>10</sup>  $R_p$ <sup>9</sup> found (38) 7.5, 7.5, 7.9 8.2 10.0, 9.3, 10.9 Mean, 8.7  
Predicted 11.6

Ovalbumin Dye— $M = 44,600$ <sup>11</sup>  $R_s$ , found (14) > 11.6, < 10.2, > 11.5  
> 9.2, 8.3, 8.6, 8.6 9.0 Mean 9.6 Predicted 11.5

Hemoglobin— $M = 69,000$  (34)  $R = R_s$ ,<sup>12</sup> found (37) 9.2 8.7, 8.6, 10.1,  
9.1, (7)<sup>12</sup> 13 9.3, 7.4  $R_{sp}$  (25) 8.2 8.2 9.2 9.5 Mean, 8.87 Predicted, 8.73

Serum Albumin— $M = 70,200$  (34)  $R_s$ , found (35) 7.5, 6.3, (26) 6.3, 8.6  
7.1, 7.8, 7.4, 8.3 Mean, 7.42 Predicted, 8.63

Serum Albumin Dye— $M = 78,400$ <sup>14</sup>  $R_s$ , found (26) 10.0 Predicted 8.06

Horse Serum Globulin— $M = 167,000$  (34)  $R_p$ , found (30) 4.5, 4.0 4.6,

<sup>4</sup>Obtained by averaging the values at the two ends of the equivalence zone

<sup>5</sup> $R = \frac{ab}{ag} = \frac{ppl}{ag} - 1$

<sup>6</sup>Omitting results with sera from young animals which according to Baumgartner give higher ratios

<sup>7</sup>Calculated from 1.53 per cent As

<sup>8</sup>Calculated from 6.2 per cent I

<sup>9</sup> $R = 100/(\text{per cent } ag) - 1$

<sup>10</sup>Calculated from 6.9 per cent I

<sup>11</sup>Calculated from eight introduced dye residues

<sup>12</sup>Using the values near the middle of the range given

<sup>13</sup> $R = 100/(\text{per cent } ag) - 1$

<sup>14</sup>Calculated from sixteen introduced dye residues

4 4, 3 8, 3 9, 3 0, 3 4, 3 6, 3 9, 3 0, 3 0, 3 2, 3 2, 3 9, 3 8 Mean, 3 70 Predicted, 5 83

*Horse-Serum-Globulin-Arsanilic-Acid* [A]— $M = 172,500^{15}$   $R_{op}$ ,<sup>14</sup> found (31) 1 53, 1 73, 7 6 Mean, 3 62 Predicted, 5 09, [B]  $M = 180,500^{16}$   $R_{op}$ , found (31) 8 5, 5 5, 5 4 Mean, 6 47 Predicted, 4 97

*Iodo-Horse-Serum-Globulin*— $M = 182,000^{17}$   $R_{op}$ , found (31), 5 5, 6 7, 8 0, 8 6, 5 4, 6 0 Mean, 6 69 Predicted, 4 93

*Edestin*— $M = 309,000$  (34)  $R_{op}$ , found (25) 1 4, 1 3, 1 2, 1 2, 1 3, 1 3, 1 8, 1 9, 2 1, 1 6 Mean, 1 51 Predicted, 3 69

*Thyroglobulin*— $M = 650,000$  (19)  $R_{eq}$ , found (32) 2 2, 2 0, 1 8, 2 5, 2 1, 3 5, 2 4, 2 7, 2 1, 3 6, 3 0, 3 0, 3 3, 2 4, 4 0, 2 2 Mean, 2 68 Predicted, 2 50

*Homarus americanus Hemocyanin*— $M = 725,000$  (34)  $R_{op}$ , found (25) 1 1, 1 0, 1 0, 0 9, 1 1, 1 0, (29) 1 2, 1 3, 0 8, 0 5, 0 3, 3 3, 2 5, 3 8, 3 3 Mean, 1 54 Predicted, 2 34

*Cancer irroratus Hemocyanin*— $M = 725,000^{18}$   $R_{op}$ , found (25) 1 8, 1 0, 2 0, 2 0, 2 6, 2 6, 1 6, 1 6, 2 3, 2 4, *species*<sup>2</sup> 1 2, 1 3, 1 7, 1 6 Mean, 1 84 Predicted, 2 34

*Limulus polyphemus Hemocyanin*— $M = ca\ 3,000,000^{19}$   $R_{op}$ , found (22) 1 4, 1 3, 0 9, 1 4, 1 3, 1 2, (25) 1 6, (28) 2 4, 2 3 Mean, 1 53 Predicted, 1 22

*Vimparus malleatus Hemocyanin*— $M = 6,630,000^{20}$   $R_{op}$ , found (28) 2 7, 2 5, 4 6, 4 0, 3 5, 2 5, 2 1, 1 6, 2 7, 2 4, 3 8, 3 2 Mean, 2 97 Predicted, 0 88

*Busycon canaliculatum Hemocyanin*— $M = 6,760,000$  (34)  $R_{of}$ , found (22) 0 7, 0 8, 0 6, 0 6, 0 6, 0 7, 0 8, 0 7 Mean, 0 68 Predicted, 0 86

## RESULTS

The above figures show that there is a strong tendency for  $R$  to be lower with the antigens of higher molecular weight. The agreement between the values of  $R$  predicted by our theory and those determined experimentally is in general close, considering the wide scatter of some of the experimental values, though there are a few conspicuous exceptions. These relations are brought out in Fig 1, where the values of  $\log R$  are plotted against the logarithm of the molecular weight of

<sup>15</sup> Calculated from 1 1 per cent *As*

<sup>16</sup> Calculated from 2 54 per cent *As*

<sup>17</sup> Calculated from 8 4 per cent *I*

<sup>18</sup> Assuming it to be the same as *Homarus*

<sup>19</sup> Svedberg states (33) that *Limulus* blood contains three hemocyanins with sedimentation constants of 57 1, 34 1, and  $16 9 \times 10^{-13}$ . The first would have a molecular weight of about 3,000,000. There is some evidence (24) that this was the component chiefly affected by the sera examined.

<sup>20</sup> Assuming it to be the same as *Helix* (34)

the antigen. The lower curve, which goes approximately through the middle of each large group of points, represents the values of  $R$  calculated from the equation

It has been stated by Eagle (9) that "there is as yet no experimental evidence that proteins are constructed of unit spheres

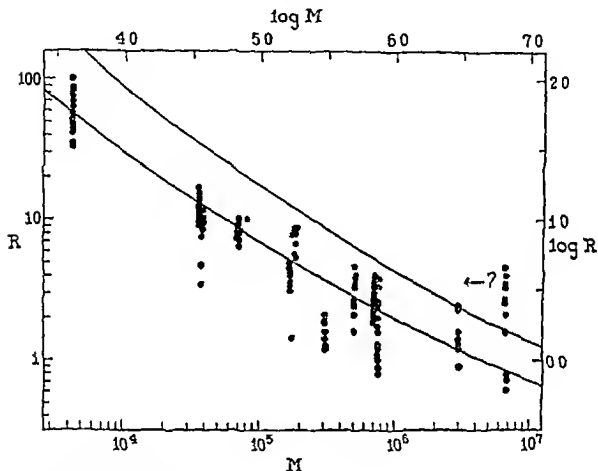


FIG 1 Relation of molecular weight of the antigen to the ratio of antibody to antigen in precipitates

Experimental values solid circles

Lower curve relation calculated assuming Svedberg unit structure of antibody

Upper curve relation calculated assuming spherical antibody (9)

The queried data at right are also plotted on abscissa  $5 \times 10^5$  for reasons discussed in the text

the experimental data are likewise compatible with the assumption that the antibody globulin is deposited as a single spherical molecule." The upper curve in the graph gives the values of  $R$  calculated according to this suggestion, using the value 150,000 (20) for the molecular weight of (rabbit) antibody. It is evident that Eagle's assumption

will give results definitely too high, unless some additional assumption is made. With the larger molecules of horse-antibody the results would be still more out of agreement.

In order to test the extent to which the experimental values of  $R$  depend upon the molecular weight of the antigen, we have calculated the regression coefficient (10), of  $R$  on  $M$ , or rather, to make the numbers involved more manageable, of  $\log R$  on  $\log M$ . We obtained a regression coefficient of  $-0.529$ , with a standard error of  $0.014$ . The coefficient is over 35 times its standard error, so there would seem to be no question that there is a highly significant degree of correlation between  $R$  and  $M$ .

To express this relation, in so far as it is linear, a straight line can be fitted to the data by the method of least squares. This gives

$$\log R = 3.49 - 0.529 \log M$$

The sum of the squares of the deviations of the observed values of  $\log R$  from those predicted from this equation is  $11.51$ . From our (curvilinear) relation we obtain  $10.29$ . Thus it appears that our theoretical equation expresses the trend of the data, not merely as well, but actually slightly better, than the best fitting straight line possible. Perhaps an empirical curved line, involving several terms, might fit better than either, but since such a curve would have no theoretical significance, we have not troubled to attempt its construction. (The empirical equation [2] connecting  $R$  and  $M$  was constructed to fit our theoretical relation, not a plot of the experimental data.)

#### DISCUSSION

That the ratio of antibody to antigen in "neutral" precipitates is strongly influenced by the molecular weight of the antigen, seems to be established by the facts presented here. It is our purpose to call attention to the fact of this relation, and the degree of it as measured by the regression coefficient. The individual determinations, however, may vary considerably, because of experimental errors and because other factors besides the size of the antigenic molecule doubtlessly influence the ratio. Among the possible factors suggested are the relative number and spacing of reactive groups in the antigen (and antibody (15)) molecules, the quality (avidity) of antibody

from a particular animal or bleeding (28), flattening of antibody, and molecular dissociation or "depolymerization" of antigen. These are now to be discussed in connection with some advantages seemingly offered by our hypothetical model of antibody antigen reactions.

Despite the predictive applicability of the equation to the relation between  $R$  and  $M$ , it is possibly fortuitous and we do not regard it as proving the literal accuracy of our model. Indeed, the agreement in the case of the pneumococcal anticarbohydrates is almost certainly accidental because the evidence now indicates that those carbohydrates are chain shaped rather than spherical. Here the fit could be ascribed to an accidentally appropriate spacing of determinant groups on the antigen or perhaps to the effect of steric hindrance to the attachment of antibody, an interference that could be more marked in the case of slender molecules than spherical ones.

The larger molecules of protein may deviate from sphericity, some considerably, but this makes surprisingly little difference in the surface volume relationships. Volumes being equal, the ratio of the surface of a prolate spheroid to that of a sphere is 1.08 when the major is twice the minor axis, and even when it is five times as long the ratio is only 1.37, with oblate spheroids the ratios are slightly higher. The appropriateness of the model depends upon two basic facts, one serological, one geometrical. (a) immunochemical combination takes place at the surface of the antigenic particle, this appears to be well established, (b) deviation from sphericity of antigenic molecules has little effect upon the amount of surface to be covered, but nonsphericity of antibody molecules enables them to cover considerably more surface. Ultracentrifugal study of antibodies (27) indicates that one axis of an antibody molecule is about a fifth as long as the others (11, ref. 12). It has been pointed out that this is a molecule having about the shape called for by our hypothesis, which postulates a chain composed of a limited number (say 4 for rabbit antibody) of contiguous, practically spherical Svedberg units. In addition to the contributions from Svedberg's laboratory relating to the physical structure of proteic molecules and their dissociation into components having an orderly range of dimensions, Bergmann and Niemann (4) offer chemical evidence that affords new reasons for accepting the fundamental reality of a unit of protein structure about the size of the



Svedberg unit, although an exactly uniform size in all proteins should not be expected. Wrinch (36) has proposed a hypothetical structure of proteins, a laminar series of cyclols, capable of leading to a unit of similar size. The idea that the unitary structure must be of limited size, in order to resist disintegration by vibrational forces, was offered by Astbury and Woods (2).

But other models can also fit the observations satisfactorily. One could assume that the antigenic molecules are coated in neutral mixtures with a layer<sup>21</sup> of antibody of the same thickness in all cases, saying nothing about the arrangement of the molecules of antibody, the results would be practically identical. Although it is now hardly permissible to regard the whole molecule of antibody as a sphere, if it is so regarded then the theoretical values of  $R$  become from 1.9 to 3.3 times too high (Fig. 1) if the antigen is completely coated. However, if the (spherical) antibody molecules are considered to be distorted somewhat by combination with the antigen, becoming flattened so as to cover more surface, then the assumption becomes workable in this part of the range. Distortion sufficient to make the major axis of the molecule twice the minor axis would be required to cover the surface completely.

Eagle has assumed that the antibody molecules are not distorted and that the surface of the antigen is not completely covered. He stated (9) "it is improbable that the chance collisions between antibody and antigen which result in combination would make for the maximum possible coverage of the antigen particle. Instead, since each antibody molecule would be bound more or less where it struck the antigen, one would expect some free space between adjacent antibody molecules, less than the diameter of each, yet constituting

<sup>21</sup>It must be noted that this thickness would have to be considerably greater than in the protein films measured by Gorter and by Langmuir where a value of 0.8–1.0  $m\mu$  was found. If we assume the molecule of ovalbumin, which has a radius of about 2.17  $m\mu$  (30  $c$ ), to be coated uniformly with a layer of protein 1  $m\mu$  thick, the ratio of antibody to antigen would be calculated as  $(3.17^3 - 2.17^3)/2.17^3 = 2.13$ , which agrees poorly with the experimental values which average 11.5. A uniform layer of protein about 3  $m\mu$  thick on the surface of the antigenic molecule would yield figures comparable to those based on our model. Perhaps if we were dealing with minimal rather than equivalent quantities of antibody the indicated thickness of the layer would be closer to 1  $m\mu$ .

a significant proportion of the total surface " But, on this assumption, it is not possible to account for the fact that the antigen is capable of combining firmly with 2 to 3 times as much antibody as that found at the optimum or at the equivalence point We, on the other hand, could attempt to explain the difference by assuming that in neutral mixtures the antibody chains apply themselves closely to the antigenic surface, whereas with excessive antibody, they "stand on their heads" that is, are attached by perhaps only one of their component units, leaving the rest projecting into space to contribute to the total amount of combined antibody but not to the effective coverage of the antigen (5) Such a picture is also consistent with the known higher dissociability of antibody from precipitates made with excessive antibody

Either the assumption of distortion or of a steric limitation respecting the portion of the antigenic surface that can be covered, virtually is mathematically equivalent to introducing an arbitrary constant into equation [1] (and using 150,000 instead of 35,200) It hardly seems plausible that the forces required for a uniform degree of flattening of the antibody molecules should be so constantly and evenly distributed on the surfaces of a large variety of antigenic proteins (or carbohydrates or lipoids) It will be noted that there is no arbitrary constant in equation [1], a fact which should lend greater significance to its agreement with the experimental data

Some objections to the lattice hypothesis have previously been discussed (24)

The agreement obtained with synthetic antigens and with edestin and *Viviparus* hemocyanin is rather poor We have included the results with chemically modified proteins (14, 26, 31), though it might well be argued that there is a distinct possibility that the molecular size might have been altered by the rather violent processing Also we do not know whether enough of the (artificial) determinants have been affixed to its surface to enable the antigenic molecule to be completely coated This is an obvious requirement for the satisfactory working of the hypothesis and certainly influences the ratio as importantly as the molecular weight (surface) of the antigen We found that casein arsenic acid must contain a minimum of about 1 per cent of arsenic in order to be precipitable with anti-ovalbumin arsenic acid (22 b) and Marrack has reported " that with a given antiserum

to *p*-amino-benzene-arsinic acid, the amount of antigen equivalent to a given volume of antiserum at optimal proportions was inversely proportional to the arsenic content of the antigen" (30 *b*)

The possible influence that the quality of the antibody may have upon the ratio is not so clear. It could be expected that highly avid antibody would be less dissociable and so give a relatively high ratio. The strength of a combining group presumably depends upon the completeness and faithfulness with which it reflects the detailed pattern of the antigenic determinant. It would seem that the (assumed) multivalence of antibody could influence (increase) the ratio only when the combining groups have a relatively weak affinity for their counterparts so that several points of union are needed to prevent dissociation. Indeed if most of the antibody in a given serum were thus multivalent then the ratio might well be *lower* than that yielded by strong univalent antibody because the latter could form a thicker layer due to its more "polar" orientation to the antigen. Upon continued immunization the later bleedings do tend to give higher ratios—the zone of equivalence is widened (15)—but this is not necessarily due to increasing multivalence of antibody, it could equally well result from the increased formation of univalent antibodies directed toward minor determinants to which the animal responds only after prolonged stimulation. A similar explanation could be considered as an alternative to the assumption that only a single immunologically reactive group is possessed by the kind of antibody that "does not precipitate antigen when separated from the rest of the antibody, but is capable of adding to a specific aggregate formed by multivalent antibody and antigen" (11). It may be that such a univalent antibody is capable of uniting with adequate firmness but

we found *Viviparus* hemocyanin might fairly have been excluded because we do not know its molecular weight, but have plausibly, we think, assumed it to be the same as that of *Helix* hemocyanin (34). But Svedberg has found that dissociation into smaller fragments is especially easy in the case of snail hemocyanins, occurring in the case of *Helix* at pH 8 or less. Some of our antisera, after storage in glass, have been found to be as alkaline as this. If such dissociation had taken place here, it might well have led to a higher ratio than we expected. In Fig 1 the ratios for *Viviparus* are plotted, to show this, against  $M = 503,000$  (a dissociation component of *Helix*) as well as against the assumed 6,630,000. The agreement for the dissociation component is seen to be good. In spite of these discrepancies, the general agreement remains striking.

#### SUMMARY

A statistical examination of the available data on the ratio of antibody to antigen in specific precipitates made at or near the optimum shows a definite correlation between the ratio and the molecular weight of antigen (regression coefficient =  $-0.529 (\pm 0.014)$ ). The authors' assumption that at this point the antigen molecules are just about covered by a layer of antibody behaving as contiguous spherical ("Svedberg") units of weight 35,200 leads to predicted ratios that in general agree well with those found, though individual experimental determinations may deviate considerably.

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# EFFECT OF THE FORMATION OF INERT PROTEIN ON THE KINETICS OF THE AUTOCATALYTIC FORMATION OF TRYPSIN FROM TRYPSINOGEN

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Vernon as early as 1901 discovered the remarkable fact that both "tryptic and rennet ferments" can be formed from their corresponding zymogens by the action of trypsin itself (1). Vernon's results were fully confirmed by Kunitz and Northrop who found that trypsin brings about the formation of chymotrypsin from crystalline chymotrypsinogen (2) as well as the formation of trypsin from crystalline trypsinogen (3). The process of formation of chymotrypsin by trypsin follows the course of a catalytic unimolecular reaction, the formation of trypsin by trypsin, on the other hand, follows the course of an autocatalytic reaction. The rate of the autocatalytic formation of trypsin is greatly enhanced in the presence of high concentrations of ammonium or magnesium sulfate.

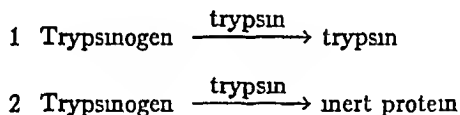
Recently Anson (4) found that trypsin brings about the catalytic formation of carboxypeptidase from its inactive precursor found in beef pancreas.

Trypsin has been found (5) to have an accelerating effect on the clotting of blood. Waldschmidt Leitz, Stadtler, and Steigenwaldt suggested (6) that trypsin acts like thrombin on fibrinogen. Eagle and Harris found (7) that crystalline trypsin coagulated citrated plasma of the rabbit, guinea pig, horse, and man. They believe that the action of trypsin is due to its catalytic transformation of prothrombin into thrombin. Mellanby and Pratt (8) disagree with the conclusions of Eagle and Harris. According to the English authors trypsin liberates active thrombokinase which acts on protrombin to form thrombin in the presence of calcium ions. As a source of trypsin

Mellanby and Pratt used pancreatic juice of cats activated by means of enterokinase

This paper describes a new enzymatic action of crystalline trypsin, namely the transformation of crystalline trypsinogen protein into an inert protein which can no longer be changed into trypsin protein either by the addition of trypsin, enterokinase, or mold kinase (9). The formation of inert protein occurs whenever crystalline trypsinogen containing a trace of trypsin is dissolved in dilute buffer solution of pH 5.0–9.0. This range of pH is also favorable for the autocatalytic transformation of trypsinogen into trypsin. Hence the presence of a trace of trypsin in a solution of crystalline trypsinogen in this range of pH brings about a gradual change of the trypsinogen partly into trypsin and partly into inert protein with the result that the process of formation of trypsin under these conditions diverges from the reaction of the simple autocatalytic type.

The two simultaneous processes may be represented schematically as follows



The first reaction, as mentioned before, proceeds in accordance with the law of an autocatalytic unimolecular reaction, namely that the rate of formation of trypsin is proportional to the concentration of trypsinogen as well as to the concentration of trypsin in solution. With regard to the second reaction, experimental studies show that the transformation of trypsinogen into inert protein is catalyzed by trypsin and that the process follows the course of a simple catalytic unimolecular reaction, the rate of formation of inert protein being proportional to the concentration of trypsin (the catalyst) as well as to the concentration of trypsinogen (the substrate).<sup>1</sup>

The relative rate of formation of the two proteins from trypsinogen depends on the pH of the solution, the rate of formation of inert protein being increased more rapidly with increase of pH than that of formation of trypsin. Hence the proportion of trypsinogen changed

<sup>1</sup> This reaction also complicates the kinetics of the formation of trypsin from trypsinogen by enterokinase (11)

into inert protein is greater in the alkaline range of pH, while in the range of pH 5.0-6.0 the proportion of trypsinogen changed into trypsin is greater.

In acid solution no inert protein is formed and hence trypsinogen may be completely transformed into trypsin by mold kinase at pH 3.0 (9).

*Theoretical Study of the Kinetics of Formation of Trypsin and Inert Protein from Trypsinogen by Trypsin*

The mathematical derivation of the equations for the kinetics of the simultaneous formation of trypsin and inert protein from trypsinogen is as follows:

Let  $G$  = original concentration of trypsinogen

$A$  = original concentration of trypsin

$A$  = total concentration of trypsin at any time  $t$

$A - A$  = concentration of trypsin formed at any time  $t$

$A$  = final total concentration of trypsin

$A - A$  = final concentration of trypsin formed

$I$  = concentration of inert protein formed in any time  $t$

$G + A - A - I$  = concentration of trypsinogen at any time  $t$

$G + A - I$  = "available activity" = trypsinogen + trypsin

$I$  = final concentration of inert protein formed

All concentrations are expressed in milligrams protein nitrogen per milliliter activation mixture.

Assuming that the rate of formation of both trypsin and inert protein is proportional to the concentration of trypsin as well as to the concentration of trypsinogen in solution we have

$$\frac{dA}{dt} = K_1 A (G + A - A - I) \quad (1)$$

$$\frac{dI}{dt} = K_2 A (G + A - A - I) \quad (2)$$

hence

$$\frac{dI}{dA} = \frac{K_2}{K_1} \quad (3)$$

and on integration

$$I = \frac{K_2}{K_1} (A - A) \quad (4)$$



Mellanby and Pratt used pancreatic juice of cats activated by means of enterokinase

This paper describes a new enzymatic action of crystalline trypsin

The values of  $\log \frac{A}{A_0 - A}$  when plotted against  $t$  should lie on a straight line, the slope of which is

$$m = \frac{K_1 + K_2}{2.3} A = \frac{K_1 G + (K_1 + K_2) A}{2.3}$$

in accordance with equation 9. The values of  $K_1$  and  $K_2$  are thus easily determinable when  $G_0$ ,  $A_0$ , and  $A$  are known. Thus

$$K_1 + K_2 = \frac{2.3m}{A} \quad (9b)$$

and

$$K_1 = \frac{(K_1 + K_2)(A - A_0)}{G} = \frac{2.3m}{G} \times \frac{A - A_0}{A} \quad (9c)$$

When  $A_0$  is negligible compared with  $A$ , then

$$K_1 = \frac{2.3m}{G} \quad (9d)$$

which also follows from equation 9a directly

Equation 5a and hence equation 9 are identical with the true equations of an autocatalytic unimolecular reaction (12), namely,

$$\log \frac{A}{A - A_0} - \log \frac{A_0}{A_0 - A_0} = \frac{K}{2.3} A t$$

except for the presence of two velocity constants and for the specific meaning of the term  $(A_0 - A)$  which does not represent the concentration of substrate at any time  $t$  as would be the case if there were no complication due to the second simultaneous reaction. (It is to be noticed that in the equation cited  $A$  is equal to  $G_0 + A$  while here

$$A_0 = \frac{K_1}{K_1 + K_2} G + A_0)$$

The expression for the time rate of formation of inert protein is obtained by substituting in equation 2 values for  $A$  in terms of  $I$  as obtainable from equation 4, namely,  $A = \frac{K_1}{K_2} I + A$  and then integrating in terms of  $I$ . A simpler solution is to substitute the expression for  $A$  in terms of  $I$  directly into the integrated equation 9

We get then the integrated equation for the time rate of formation of inert protein, namely,

$$\log \frac{K_1 I + K_2 A_0}{I_e - I} - \log \frac{K_2 A_0}{I_e} = \frac{K_1 + K_2}{2 \cdot 3 K_2} (K_1 I_e + K_2 A_0) t \quad (10)$$

which is similar to equation 9, the plotted curve of  $I$  vs  $t$  should resemble an S shaped curve typical for an autocatalytic unimolecular reaction

The derived equations bring out the following quantitative relationships between the two products formed from trypsinogen by means of trypsin under constant conditions of pH, temperature, and salt concentration

1 The concentration of inert protein in the reaction mixture at any time  $t$ , as well as at the end of the reaction, is proportional to the concentration of trypsin formed<sup>2</sup> It follows that the specific activity, *z e* the number of tryptic units per milligram protein nitrogen, of the final product is constant and is independent of the original concentration of trypsinogen

2 The final concentration of trypsin formed as well as the final concentration of inert protein formed is proportional to the original concentration of trypsinogen and is independent of the original concentration of trypsin (equations 7 and 8) The rate of formation of these products is, however, dependent on the original concentration of trypsin in the reaction mixture

3 The equation for the rate of formation of trypsin still remains autocatalytic in form (equation 9) although it is slightly modified by the correction due to the formation of inert protein

4 The equation for the rate of formation of inert protein is, like the one for the rate of formation of trypsin, of an autocatalytic form (equation 10)

<sup>2</sup> All the derived equations would still hold true were inert protein instead of trypsin assumed to be the catalyzing agent in both reactions, since the concentrations of the two products are proportional to each other The experiments, however, on the effect of varying the initial concentration of trypsin show definitely that the rate of formation of both products is proportional to the concentration of trypsin It follows, therefore, that trypsin is the catalyst in both reactions

All these relationships have been found to check closely with the experimental facts

*Experimental Studies of the Kinetics of Transformation of Crystalline Trypsinogen into Trypsin and Inert Protein*

**General Procedure**—Solutions were made of crystalline trypsinogen in dilute buffers and allowed to stand at 5°C.<sup>3</sup> In most experiments it was unnecessary to add trypsin since the crystalline trypsinogen used generally contained small but measurable amounts of trypsin which were sufficient to initiate the reactions. Duplicate samples of 1 ml were taken at various times. One set of the 1 ml samples was acidified with hydrochloric acid to about pH 2.0 in order to stop the reactions. The concentration of trypsin in these samples was then determined by the hemoglobin method of Anson (10). These measurements provided the data for the rate of formation of trypsin. The 1 ml samples of the other set were mixed with equal amounts of a concentrated solution of enterokinase (200 [E.K.U.]/ml) made up in  $\pi/10$  pH 7.6 phosphate buffer and placed at 5°C. This brought about very rapid transformation of all of the available trypsinogen protein into trypsin protein without any formation of inert protein as will be described in a later paper on enterokinase (11). The samples were acidified with hydrochloric acid to pH 2.0 after 2 hours and the concentration of trypsin in these samples was determined. If there were no gradual formation of inert protein in the trypsinogen solution then the concentration of trypsin (available activity) in the enterokinase activated samples taken at various times would remain constant and would be numerically equal to the concentration of trypsin added to or present initially in the trypsinogen solution plus the original concentration of trypsinogen. Actually it was found that in all cases where the pH of the trypsinogen solution was above 4.0 there was a gradual lowering of the concentration of available tryptic activity in the successive samples. This gradual loss in available activity was due to the formation of inert protein since the total concentration of protein in the trypsinogen solution, as measured by the amount of precipitate formed with 2.5 per cent trichloroacetic acid remained practically constant up to the end of the reaction.

The rate of formation of inert protein in the original reaction mixture was calculated as follows

Let  $P$  = initial available activity, and

$P_t$  = available activity at any time  $t$ . The concentration of inert protein formed at any time  $t$  is then  $I_t = P - P_t$ .

<sup>3</sup> It was found necessary throughout these studies to keep all reaction mixtures at a temperature not higher than 5°C in order to avoid complications due to protein hydrolysis by trypsin.

The solutions were kept sterile by the addition of 0.1 ml 1 per cent merthiolate in 1.4 per cent borax solution to 100 ml of reaction mixture.

An experiment illustrating the various measurements is given in Table I. The concentration of trypsin as well as the concentrations of available activity are expressed both in tryptic units  $[T U]_{ml}^{Hb}$  and also in milligrams protein nitrogen per milliliter. The last were obtained by dividing the  $[T U]_{mg P N}^{Hb}$  by 0.17 which is the specific activity, *i.e.*,  $[T U]_{mg P N}^{Hb}$  of pure trypsin.

The experiment shows the following

- 1 The final concentration of trypsin formed is only about one-fourth of the initial available activity
- 2 The total available activity is diminished rapidly with time until it equals the concentration of trypsin in solution

TABLE I

*Formation of Trypsin and Inert Protein from Trypsinogen at pH 8.0 and 5°C*

Activation mixture: 10 ml of solution of crystalline trypsinogen in M/200 hydrochloric acid containing 1 mg protein nitrogen per ml plus 4 ml M/2 phosphate pH 8.0 plus water to 100 ml

Time at 5°C	Trypsin		Available activity		Inert protein (by difference)	Total protein measured
	$10^{-3} [T U]_{ml}^{Hb}$	$10^{-3} mg P N / ml$	$10^{-3} [T U]_{ml}^{Hb}$	$10^{-3} mg P N / ml$		
0	0.35	2.0	15.60	90	0	88
3.5	1.22	7.0	11.10	64	26	83
7.0	2.32	13.5	6.08	35	55	88
11.5	3.07	18.0	4.04	23	67	83
23.0	3.70	21.0	3.75	22	68	64
47.0	3.46	20.0	3.50	20	70	49
72.0	3.08	18.0	3.12	18	72	43

3 The total protein concentration is practically unchanged during the time of formation of trypsin, this shows that the rapid loss in available activity is due to the formation of inert protein which can no longer be activated by the excess of enterokinase used.

4 A gradual loss in protein concentration begins at the time when the trypsin concentration of the solution has reached its maximum value. This loss is due to the gradual hydrolysis by the formed trypsin of both the inert protein as well as of the trypsin itself. This gradual destruction of protein by the trypsin is greatly reduced if the pH of the solution is kept below 7.0 and the total concentration of protein below 0.1 mg protein nitrogen per milliliter.

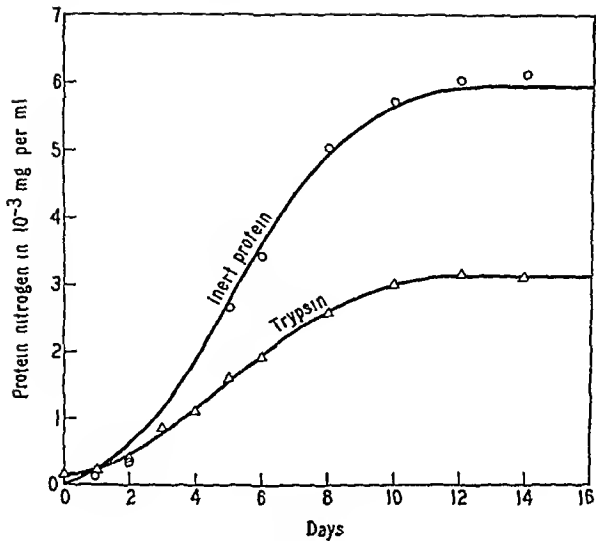


FIG 1a

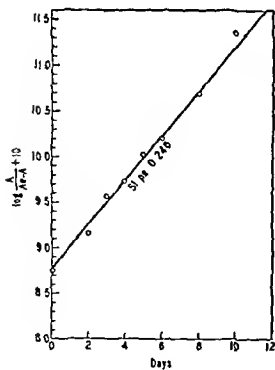


FIG 1b

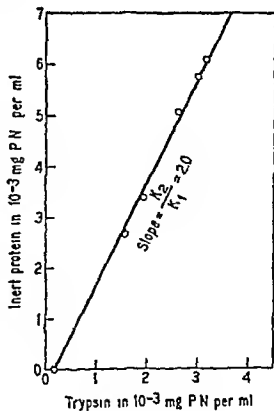


FIG 1c

FIGS 1a 1b, 1c Formation of trypsin and inert protein at pH 5.8 and 5°C  
5 ml crystalline trypsinogen, 0.1 mg protein nitrogen per ml in 0.005 M HCl

*Transformation of Trypsinogen into Trypsin and Inert Protein*

Fig 1 *a* shows graphically the experimental data for the time rate of formation of trypsin and inert protein at pH 5.8. The plotted experimental points for the rate of formation of trypsin as well as for the formation of inert protein fall within the experimental error on smooth theoretical S shaped curves. The theoretical values of  $A$  were obtained by means of the exponential form of equation 9a, namely,

$$A = A_0 \frac{\frac{A_0}{A_0 - A_0} e^{\kappa_1 G_0 t}}{1 + \frac{A_0}{A_0 - A_0} e^{\kappa_1 G_0 t}}$$

The theoretical values of  $I$  were calculated from the theoretical values of  $A$  by means of equation 4, namely,

$$I = \frac{K_2}{K_1} (A - A_0)$$

The values for  $K_1$  and for  $\frac{K_2}{K_1}$  were calculated from the slopes of the rectilinear curves in Figs 1 *b* and 1 *c*.

Fig 1 *b* shows the plotted points of  $\log \frac{A}{A_0 - A}$  vs  $t$ . The experimental points fall on a straight line in accordance with equation 9. The proportionality relation between the values of  $A$  and  $I$  is shown in Fig 1 *c* in agreement with equation 4.

*Effect of Varying the Concentration of Trypsinogen on the Kinetics of Formation of Trypsin and Inert Protein*

Equation 9 *a* predicts that for various values of initial concentration of trypsinogen the slope of the plotted rectilinear curve of  $\log \frac{A}{A_0 - A}$  vs  $t$  should be proportional to  $G_0$ , provided the value of  $A_0$  is small compared with  $A$ .

Fig 2 *a* shows the plotted values of  $\log \frac{A}{A_0 - A}$  vs  $t$  of a series of concentrations of trypsinogen at pH 5.8. The experimental points

lie in all cases on straight lines, the slopes of which are approximately proportional to the initial concentrations of trypsinogen used

Fig 2 *b* shows that the linear relation between  $I$  and  $A$  is independent of the initial concentration of trypsinogen, in agreement with equation 4

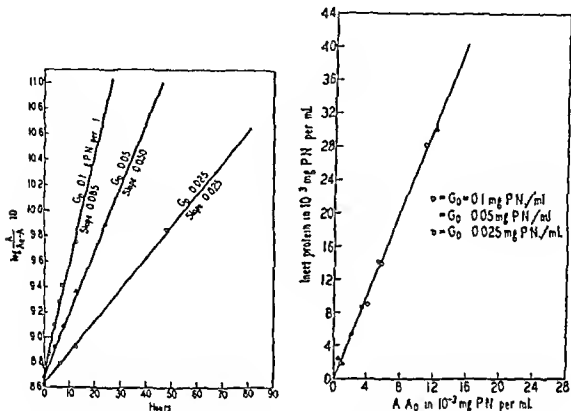


FIG 2a

FIG 2b

FIGS 2a and 2b Effect of concentration of trypsinogen

10 ml 0.1 M K-Na phosphate buffer pH 5.8 plus 50.25, or 1.25 ml crystalline trypsinogen 1 mg protein nitrogen per ml, plus 0.2 M NaOH to adjust pH to 5.8 plus water to 50 ml

#### *Effect of Varying the Initial Concentration of Active Trypsin in Solution*

The effect of varying the concentration of active trypsin added to a solution of trypsinogen of pH 5.0 is shown in Fig 3a where the concentration of trypsin formed ( $A - A_0$ ) was plotted against  $t$ . It is evident from the curves that the initial rate of formation of trypsin is proportional to the initial concentration of active trypsin in the activation mixture. The final concentration of trypsin formed and hence also the final concentration of inert protein formed is, how



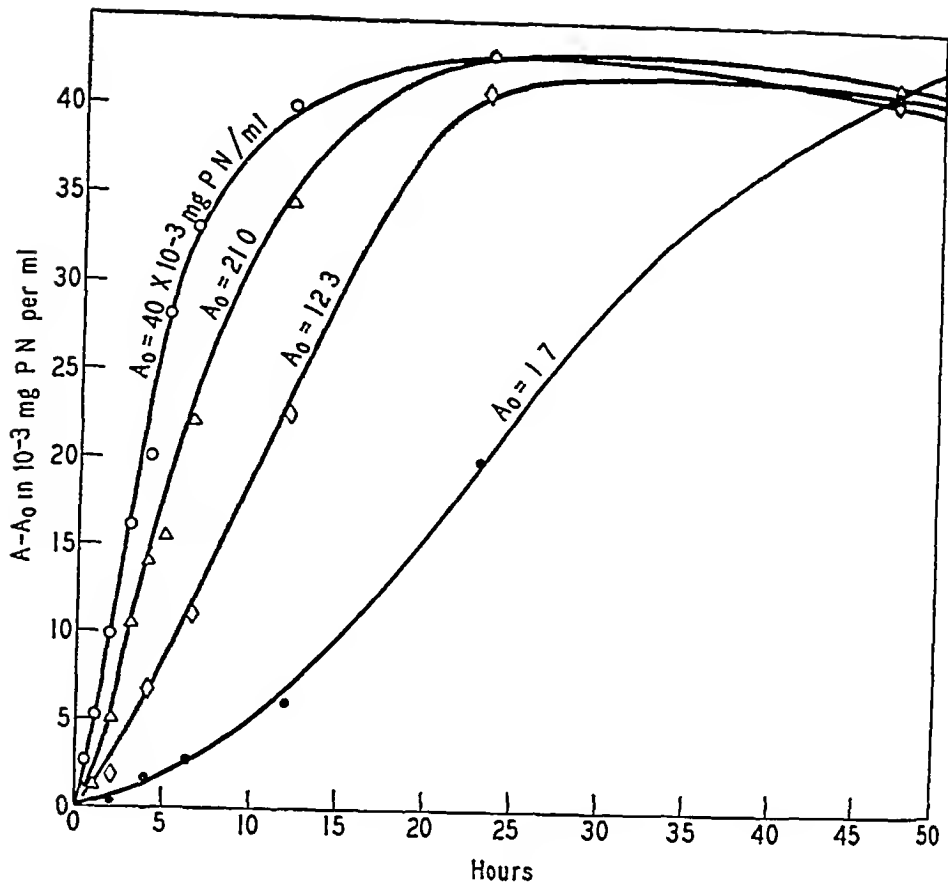


FIG 3a

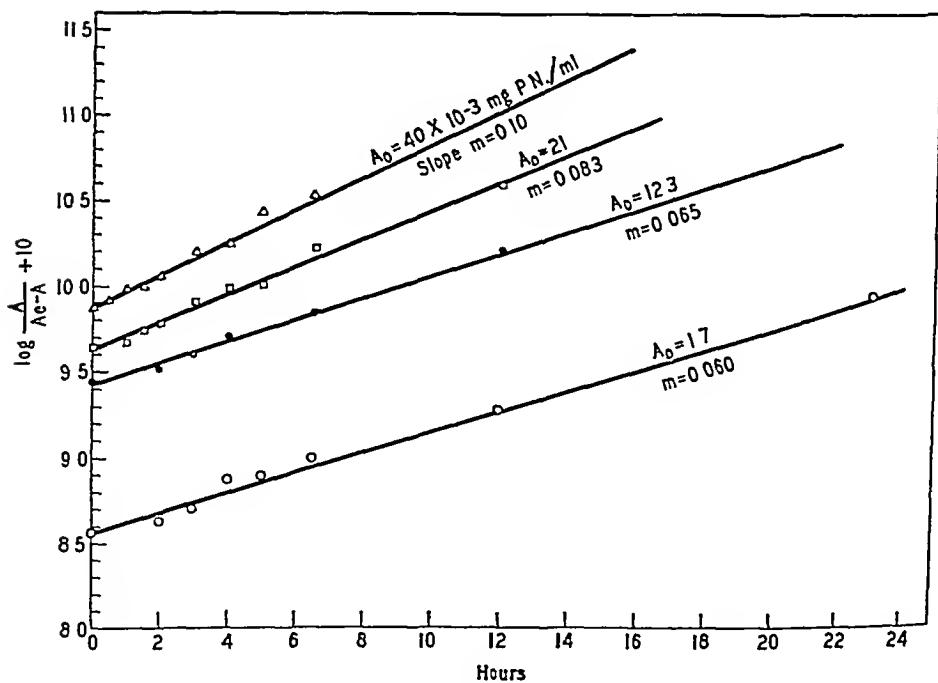


FIG 3b

FIGS 3a and 3b Effect of varying initial concentration of trypsin  
 5 ml crystalline trypsinogen, 1 mg protein nitrogen per ml in  $M/200$  HCl, plus 0, 5, 10, or 20 ml crystalline trypsin, 0.1 mg protein nitrogen per ml in 0.05  $M$  K-K phosphate buffer pH 5.0, plus 20, 15, 10, or 0 ml 0.05  $M$  K-K phosphate buffer pH 5.0 plus water to 50 ml



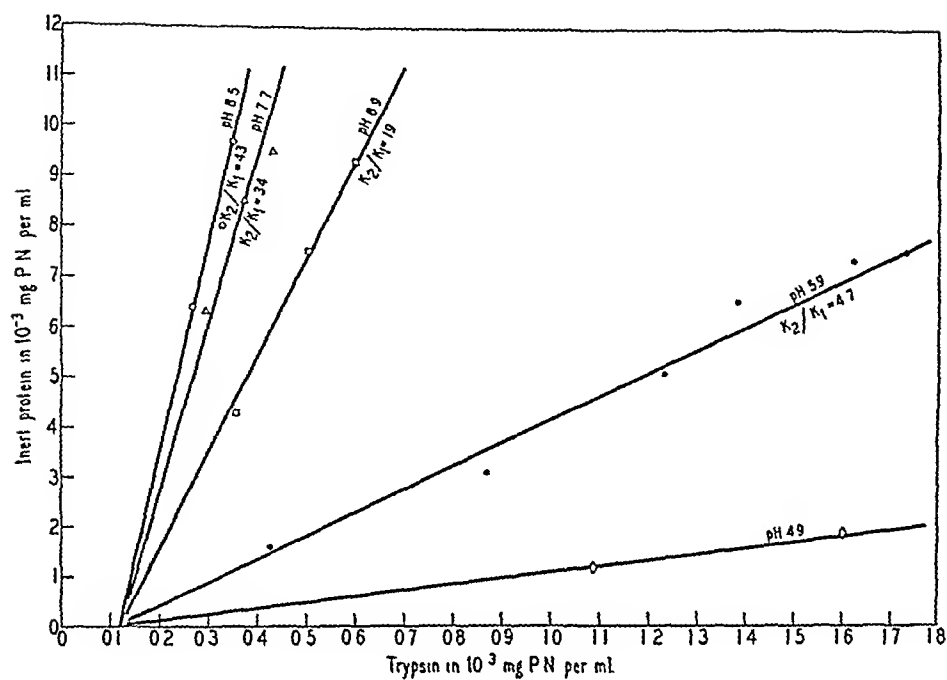


FIG 4d

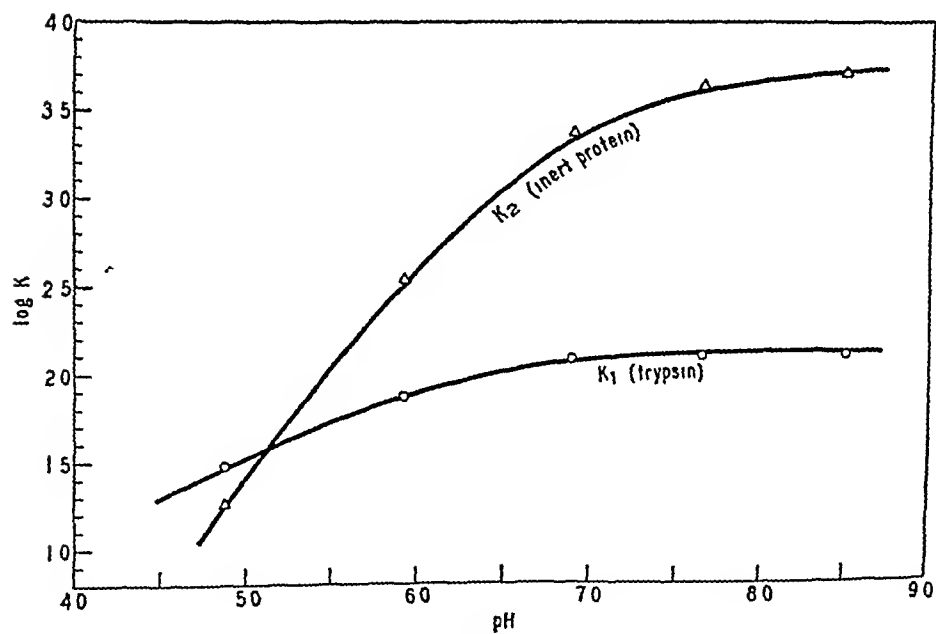


FIG 4e

equal while at lower pH the rate of formation of inert protein becomes negligible

The striking difference in the ultimate amount of trypsin formed at pH above 7.0 as compared with that formed at pH below 6.0 may be the explanation of the disagreement between Vernon, on one side, and Bayliss and Starling on the other side in regard to the question whether trypsin itself is able to convert trypsinogen into trypsin. Vernon worked with inactive pancreatic extracts and had no difficulty in activating it by means of trypsin while Bayliss and Starling employed pure pancreatic juice and found that trypsin not only did not activate the pancreatic juice but just the opposite occurred, namely, that it "destroyed the pro ferment without apparently at any time converting it into ferment" (13). Bayliss and Starling have evidently obtained results comparable to the one represented in Fig. 4a by the curve of pH 6.9 or pH 7.7 while Vernon's results correspond to the curve of pH 4.9. A difference of one or two units between the pH of the pancreatic extract used by Vernon and that of the pancreatic juice used by Bayliss and Starling would amply explain their disagreement.

The writer was assisted by Margaret R. McDonald

#### SUMMARY

A solution of crystalline trypsinogen in dilute buffer containing a trace of active trypsin when allowed to stand at pH 5.0-9.0 and 5°C is gradually transformed partly into trypsin protein and partly into an inert protein which can no longer be changed into trypsin either by enterokinase or mold kinase.

During the process of formation of trypsin and inert protein the ratio of the concentrations of the two products in any reaction mixture remains constant and is independent of the original concentration of trypsinogen protein. This ratio varies, however, with the pH of the solution, the proportion of trypsin formed being greater in the acid range of pH.

The experimental curves for the rate of formation of trypsin, as well as for the rate of formation of inert protein are symmetrical S shaped curves closely resembling those of simple autocatalytic reactions.

The kinetics of formation of trypsin and inert protein can be explained quantitatively on the theoretical assumptions that both reactions are of the simple unimolecular type, that in each case the reaction is catalyzed by trypsin, and that the rate of formation of each of the products is proportional to the concentration of trypsin as well as to the concentration of trypsinogen in solution

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# THE FLICKER RESPONSE FUNCTION FOR THE TURTLE PSEUDEMYIS

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## I

The flicker response curve has been determined for the turtle *Pseudemys*, using the technic and procedure employed in our earlier studies with insects, fishes, newts, and man<sup>1</sup> The structure of the curve, and the relations of its parameters to temperature, show several interesting features Mean critical intensities ( $I_m$ ) have been measured at constant temperature as a function of flash frequency  $F$ , with equality of light time and dark time in a flash cycle, reciprocally,  $F_m$  was obtained as a function of  $I$  The determinations of  $I_m$  were made at various temperatures The retina of *Pseudemys* contains no "rods" or so small a number that they have not been detected There is thus an opportunity to test the adequacy of equations employed for the description of the flicker contour, the performance of the peripheral sensory field of the retina is not complicated by the duplexity of receptor constitution found in vertebrates hitherto studied,<sup>2</sup> hence a single function is available for examination over the whole explorable range

The variability of performance of these turtles proves to be so slight that persisting individual differences in excitability are easily recognized among the ten specimens used The group tested is thus not really a homogeneous group, as this has been defined with certain other organisms<sup>3</sup> An experimental test of several features of the

<sup>1</sup> Wolf and Zerrahn Wolf, 1935-36, Crozier, Wolf and Zerrahn Wolf, 1936-37 a, b, c, d, 1937-38 a, b, c, d, e 1938 a, b, c, d, e

<sup>2</sup> 1938 a etc.

<sup>3</sup> 1936-37 a, b 1937-38 a

general treatment of the variability<sup>4</sup> of performance in response to visual flicker is therefore possible

Young individuals of *Pseudemys scripta* ssp., of carapace length 3.5 cm., were kept in a large terrarium near a south window, with access to water. They were fed regularly with prepared turtle food. Their activity depends upon the temperature and the light conditions, on cloudy days they are almost motionless which has some influence upon their behavior during the tests at such times. A numbered series of 10 was selected from a group of 24 on the basis of general reactivity.

For observation each turtle was put in a glass jar 10 cm. in diameter with just enough water to permit an easy foothold and yet to permit extension of the head above water for breathing. If too much water is used the animals move about and the recognition of the threshold responses may become almost impossible. The jars are placed in a water thermostat (Stier and Crozier 1932-33) for at least 1 hour, in darkness, before the measurements are made. At temperatures between 21.5° and 29° the reactions are increasingly sharp and clear as the temperature is made higher. Above 30° general activity increases, and it is necessary to wait for intervals of quiescence in which the occurrence of the index responses can be recognized with the best certainty. Below 21.5° the responses are markedly slower, but quite definite. Care is required that the rate of increase of intensity up to the critical point (or the reduction of flash frequency in the converse experiment) be not so rapid that mechanical overshooting of the end-point becomes a factor, due to the latency of the response. The determinations are made by placing the jar containing a turtle inside the striped cylinder of the apparatus,<sup>1</sup> which is set in motion at a fixed and controlled speed providing the desired flash frequency. The diaphragm governing the intensity of illumination is then slowly opened until an intensity is reached at which response occurs. The turtle being up to this point quiet, the threshold response to flicker consists in a sudden deflection of the head, in a direction against that of the motion of the stripes. This reaction may be of slight amplitude and of brief duration, subsequently, the head turns in the direction of motion of the opaque stripes and then jerks back to its original position. Some more active individuals show swimming movements following the turning of the head, while others give only the latter response. In some cases, especially at low illuminations, there may be shown only a rotation of the head about the body axis, this occurs most easily when the head is in contact with the wall of the container, and a gentle touch is used to move the turtle away from the wall. The occurrence of the first motion of the head is in any case taken to signalize that the critical flash intensity  $I_c$  has been found.

When  $F_c$  is measured as a function of fixed flash illumination  $I$  the animal is put in the apparatus with the selected illumination already turned on and with the cylinder rotating to give a flash frequency much above that critical for flicker, and

<sup>4</sup> Cf. Crozier, 1935. Crozier, Wolf, and Zerrahn-Wolf, 1936-37 a, 1937-38 a, etc.

thus a "uniformly illuminated" field. There is usually some initial activity, which quickly subsides. When the speed of rotation of the cylinder is slowly decreased the critical speed is recognized by the same response as in the case of increasing intensities with  $F$  fixed. The reactions are slow, however, and the decrease of the flash frequency must be produced smoothly and not too fast to avoid overshooting of the end point. If the critical point is much overrun the animals may be put into a state of tonic immobility from which they can be aroused only by mechanical stimulation after the light has been turned off.

Regularity and reproducibility of results, as in all such experiments, depend to a large extent upon the regularity with which the animals are fed, handled, and manipulated during use.

## II

At various fixed flash frequencies  $F$  the mean critical flash intensities  $I_m$  were measured at two temperatures, 21.5° and 29.5°, the flash cycle used has equal light and dark time ( $t_L = t_D$ ). These data are given in Table I, each value of  $\log I_m$  being accompanied by the  $\log P.E.$  of the dispersion of the ten individual mean values from which the  $I_m$  is computed. The general order of magnitude of the scatter of the critical intensities is not very different from that encountered in corresponding experiments with other forms,<sup>1</sup> certain differences in the nature and properties of this  $P.E.$ , as a function of  $I_m$  are considered subsequently.

The measurements in Table I are plotted in Fig. 1. It is apparent that, in contrast with the flicker response contours obtained from sundry other vertebrates thus far studied,<sup>2</sup> the observations fall upon a single smooth curve. They exhibit no separation into two segments, such as there has been reason to suppose with other forms represent the results of the activation of two distinct classes of irritable elements, reasonably identified with those for which respectively the excitability of rods and cones are to be held responsible.<sup>3</sup>

The histological evidence shows that the retinas of at least some diurnal reptiles contain no recognizable rods, or at least extremely few. The visual cells are of the "cone" type, but a certain proportion are "double cones." The tortoises (*Chelopus*, *Chrysemys*, etc.) and various lizards are said to have only retinal cones (Detwiler, 1916, 1923, Detwiler and Laurens, 1920, Menner, 1928, Verner, 1935, 1937). The presence of a small number of rods in the turtle retina has been stated by Walls (1934). Examination of sections of light



adapted and dark adapted retinas of our *Pseudemys* shows only one general type of visual cell, cones, these are of the usual two kinds, single and "double"

These turtles were chosen for our observations because of the "rod-free" character of the retina Unless the functional activity of the

TABLE I

*Mean Critical Intensities of Illumination ( $I_m$ ), Milhlamberts, and the Probable Errors of the Dispersions of  $I_1$ , for Response to Flicker by the Turtle *Pseudemys scripta*, at Two Temperatures, As a Function of Flash Frequency ( $F$ )*

$F/sec$	21 5°C		29 5°C	
	$\log I_m$	$\log PE_{I_1}$	$\log I_m$	$\log PE_{I_1}$
1	$\bar{6}.8221$	$\bar{7} 2472$		
2	$\bar{5} 3412$	$\bar{7} 9768$	$\bar{6} 8184$	$\bar{7} 0704$
3	$\bar{5} 7348$	$\bar{6} 1658$		
5	$\bar{4}.1635$	$\bar{6} 7687$	$\bar{5} 5760$	$\bar{7} 8397$
7 5	$\bar{4}.6103$	$\bar{5} 1402$	$\bar{4} 0803$	$\bar{6} 4183$
10	$\bar{3} 0183$	$\bar{5} 4196$	$\bar{4} 4966$	$\bar{5} 0090$
	$\bar{3} 0174$	$\bar{5} 3191$		
15	$\bar{3}.6645$	$\bar{5} 9768$	$\bar{3}.1396$	$\bar{5} 3529$
20	$\bar{2}.2036$	$\bar{4} 9146$	$\bar{3} 6764$	$\bar{5} 9764$
25	$\bar{2} 7097$	$\bar{3} 3579$	$\bar{2} 1784$	$\bar{4} 6392$
	$\bar{2} 7016$	$\bar{3} 0671$		
30	$\bar{1}.2660$	$\bar{3} 9348$		
	$\bar{1} 2368$	$\bar{3} 5710$	$\bar{2} 7036$	$\bar{3} 1623$
	$\bar{1} 2310$	$\bar{3} 3512$		
35	$\bar{1} 7569$	$\bar{2} 1804$	$\bar{1} 2240$	$\bar{3} 5637$
40	$0 2803$	$\bar{2} 8352$	$\bar{1} 7541$	$\bar{2} 0484$
45	$0 8859$	$\bar{1} 0990$	$0 3907$	$\bar{2} 8762$
48	$1 4155$	$\bar{1} 4043$	$0 8968$	$\bar{1} 2190$
50	$2.1477$	$0 3478$	$1 5973$	$\bar{1} 9853$
51			$2 1732$	$0 2762$

double cones should be different from that of the typical cones, it might then be possible to find a curve of visual performance not exhibiting the composite nature encountered with other vertebrates studied,<sup>2</sup> which has been attributed to the duplexity of the retinal constitution<sup>2</sup> It is to be recognized, of course, that the histological criteria for the distinction between rods and cones are rather un-

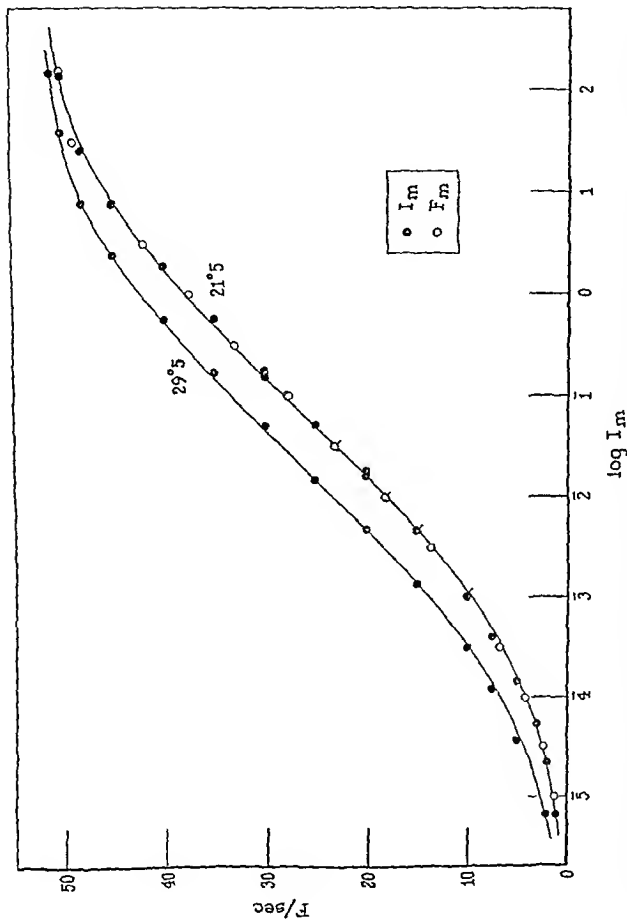


FIG. 1. Log  $I_m$  as a function of flash frequency  $F$  (solid circlets), at two temperatures ( $21.5^\circ$ ,  $29.5^\circ$ ), for *Pseudemys scripta*, data in Table I and  $I_m$  (open circlets) as a function of log  $I$  at  $21.5^\circ$  (Table II). Each plotted point is the mean of 30 measurements, 3 on each of the same 10 individuals at all points (overlapping points carry a tag). The curve drawn is the same at each temperature, a probability integral with  $F_{max} \approx 52.63$ ,  $\sigma_{log I} \approx 2.062$ ,  $\tau \approx 2.27$  ( $29.5^\circ$ ) and  $2.82$  ( $21.5^\circ$ ). The departures are slight (0.5 per cent at most), but are systematic: their origin is discussed in the text.

satisfactory and difficult to apply in a uniform way. The variations of human visual properties with location of the test spot on the retina<sup>5</sup> are indicative of a *qualitative* correlation with the associated proportions of rods and cones, and support the dissection of the visual performance curves into two portions.<sup>5</sup> Data on visual responses initiated by a rod-free retina should provide a conclusive test of the propriety of this procedure (particularly if supplemented by observations with cone-free animals). The precise determination of the form of the performance function in such a case is an important check upon the method of separation of presumptive rod and cone effects in the composite curves, especially if it appears that the measurements with the rod-free animal indicate a single population of sensory effects.

The curves drawn in Fig. 1 are computed probability integrals,

$$F = k F_{max} \int_{-\infty}^{\log I} e^{-(\log I/I_0)^2/2\sigma^2} d \log I \quad (1)$$

with the same value of  $F_{max}$  ( $=52.63$ ) for the two temperatures, and the same value of  $\sigma_{\log I}$  ( $=2.062$ ). The abscissa at the inflection point decreases with rise of temperature, as found previously<sup>2</sup> for insects and fishes. We have used in some earlier papers a logistic of the form

$$F = F_{max} / (1 + e^{-\rho \log I}) \quad (2)$$

to describe such data, because of its formal identity with the general expression for the photostationary state,<sup>6</sup> although pointing out that the parameters in the latter expression do not have properties permitting rational application to the flicker contour.<sup>6</sup> It was also pointed out<sup>7</sup> that the probability integral gives a possibly better description of the data. For theoretical reasons<sup>7</sup> equation (1) is preferred. The simple character of the *Pseudemys* flicker curve permits a formal decision between (1) and (2), since a single function appears to cover the entire range of the data. Equation (1) gives a decidedly superior fit. It is also to be noted that the data in Table I cannot be described by a photostationary state equation, moreover,

<sup>5</sup> Cf. Hecht, 1937

<sup>6</sup> 1936-37 c, d, 1938 c, 1937-38 d, e

<sup>7</sup> Crozier, 1937, 1937-38 d

the shift with temperature change (Fig 1, and Section IV) is in the reverse direction to that called for by the nature of this formulation<sup>6</sup>

### III

The departures of the  $I_m$  values from the curves drawn are slight, but are consistent and significant. Moreover, at 21.5°, the departures

TABLE II

Mean Critical Flash Frequencies ( $F_m$ ), 21.5 with P.E., at Fixed Flash Illuminations ( $\log I$ )

Because of the persisting individual differences the mean values of P.E., have been computed (cf Fig 10)

$\log I$	$F_m/\text{sec}$	P.E., $P_1$	P.E., $P$
$mL$			
$\bar{5} 0$	1 19	0 0134	0 0162
$\bar{5} 5$	2 30	0 0134	0 0198
$\bar{4} 0$	4 19	0 0606	0 0554
$\bar{4} 5$	6 76	0 0321	0 0835
$\bar{3} 0$	9 82	0 0855	0 156
$\bar{3} 5$	13 64	0 0784	0 135
$\bar{2} 0$	18 04	0 0989	0 0833
	18 14	0 103	0 183
$\bar{2} 5$	23 13	0 111	0 205
	23 10	0 176	0 282
$\bar{1} 0$	27 84	0 155	0 221
	27 61	0 137	0 212
	27 82	0 171	0 304
$\bar{1} 5$	32 94	0 226	0 393
0 0	37 43	0 2147	0 353
0 5	42 01	0 201	0 324
1 0	48 02	0 291	0 304
1 5	48 85	0 153	0 280
	48 95	0 282	0 344
2 2	60 01	0 201	0 594

of the  $F_m$  data (Table II) at fixed intensities are of the same kind. This could arise from one or several different causes: the probability integral may not really describe the data, or the averages  $I_m$  may be systematically influenced by persistent individual differences among the curves for the ten turtles, or the readings of critical intensities may suffer from systematic instrumental errors.

The existence of definite differences among the individuals used is easily demonstrated. In each series of tests ( $I_m$  at fixed  $F$ ,  $21.5^\circ$  and  $29.5^\circ$ ,  $F_m$  at fixed  $I$ ,  $21.5^\circ$ ) rank order numbers are assigned in the sequence of decreasing sensitivities in each set of readings<sup>8</sup>. This means that the rank numbers 1 to 10 are given in the order of increasing critical intensities for response, at fixed flash frequencies, and in the order of decreasing critical  $F$ , at fixed intensities. In a homogene-

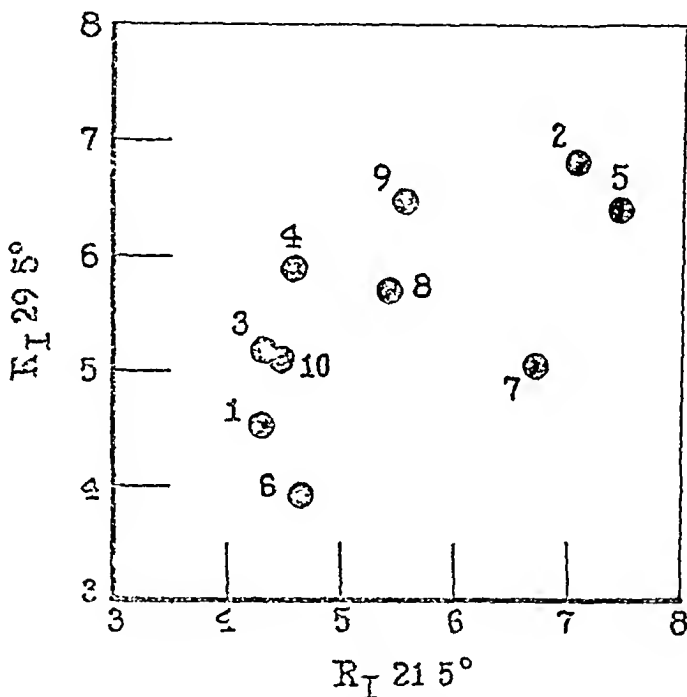


FIG 2 Mean rank order numbers for *Pseudemys* 1 to 10 at all flash frequencies, showing correlation between relative excitabilities at two temperatures

ous lot of individuals the rank order numbers for the lot are randomly distributed and there are no statistically significant differences between the mean rank numbers of the individuals<sup>8</sup>. In the *Pseudemys* experiments, however, the successive rank positions of an individual are correlated. The mean rank numbers for two individuals may differ by seven or more times the P E of the difference. Figs 2, 3, and 4 show the correlation between the mean rank numbers for the

<sup>8</sup> 1936-37 a, b, 1937-38 a

separate individuals in different tests. Fig 2 is based on the sets of observations at 21.5° and 29.5°, Fig 3 on the  $I_m$  and  $F_m$  experiments at 21.5°, and Fig 4 on the determinations of  $I$  at two flash frequencies ( $F = 20, 30$ ) at eleven temperatures between 12.2° and 35.8° (Section IV). It is apparent that there is a notable correlation between the mean rank order numbers of an individual in the various tests. The relative excitabilities of the individuals are retained over a period of about 4 months.

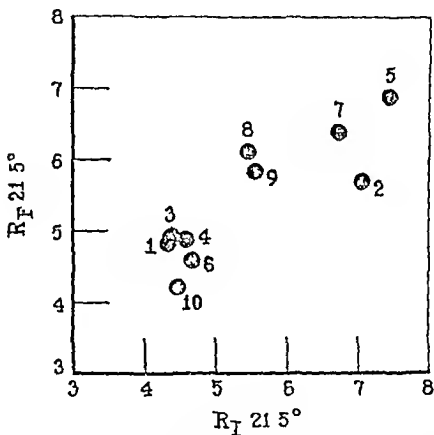


FIG 3 Showing correlation between mean rank order numbers of individuals for determinations of  $I_m$  and of  $F_m$  at one temperature

This individuality is further seen in the slight but definite and repeated differences between individuals in the values of  $\Gamma_m$  and of  $\sigma_{100}$ , which must be taken to obtain reasonable rectilinearity upon a probability grid. Fig 5 gives data for two individuals of nearly identical mean rank order numbers, at two temperatures, which illustrate this.

While differences of this sort could easily produce the kind of slight deviation from theory which Fig 1 exhibits in the averaged measure

ments, it is also true that such deviations appear in the data of practically all the individuals. They seem to be correlated with the use of particular lamps and filters to produce the different levels of intensity of illumination. Fig 6 gives illustrations. Similar deviations occur in the  $F_m$  data (Fig 7). The maximum single departure

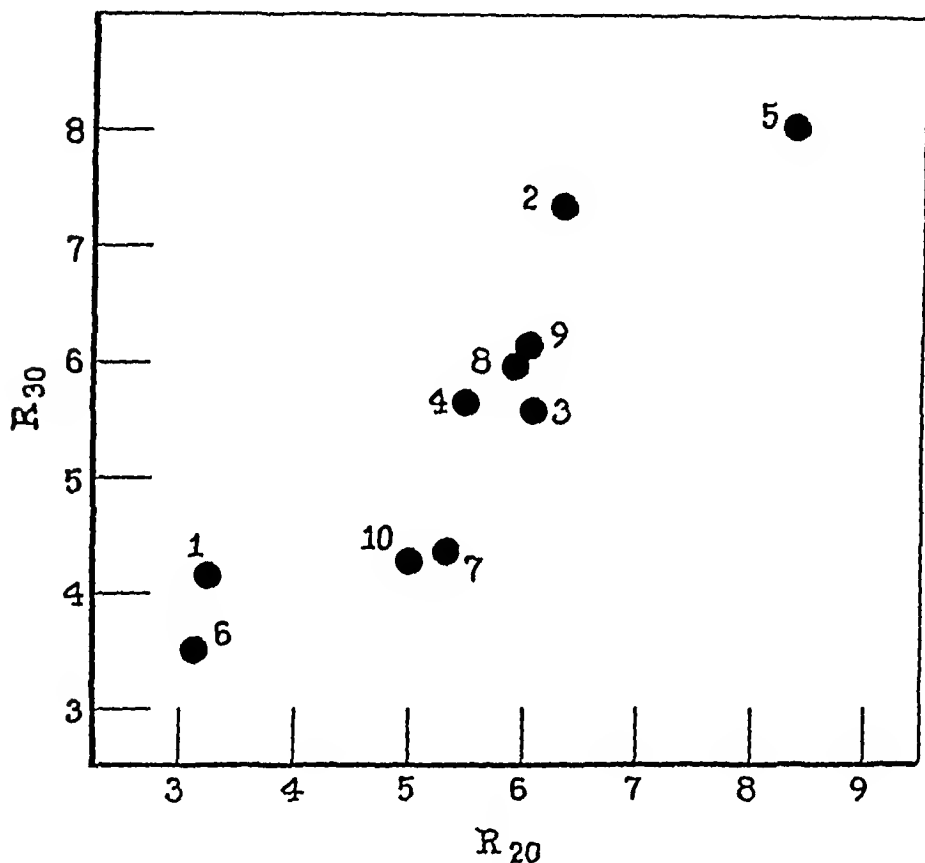


FIG 4 Showing correlation between mean rank order numbers of the individuals in determinations of  $I_m$  at various temperatures (Table III) and at two flash frequencies ( $F = 20$ ,  $F = 30$ )

is not over 0.06 log unit, except at the extreme upper and lower ends of the graphs. At the low intensity end it becomes difficult to make accurate observations while at the upper end it is hard to control intensity with adequate precision, in either case "overshooting" of the end-point is likely to be a factor.

The scatter of  $I_1$  bears the same kind of relationship to  $I_m$  as we have already found with other forms<sup>1</sup> The proportionality is direct (Fig 8) There is no real evidence of a "break" in the plot, such as is found for vertebrates having both rods and cones<sup>1</sup> There is no change of the level of P.E.<sub>11</sub> with long continued practice The order of

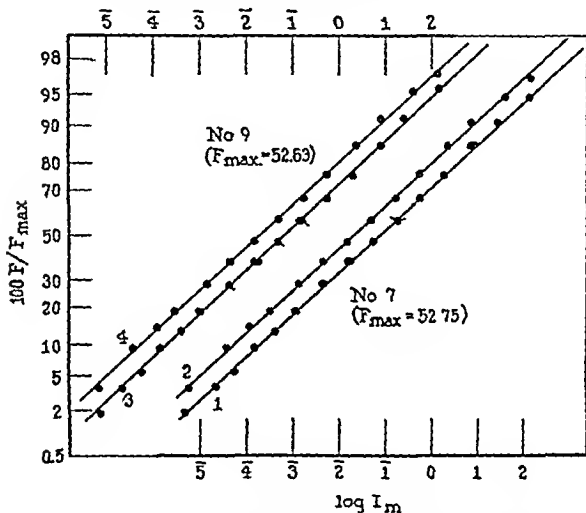


FIG 5 Showing slight but characteristic differences between the maxima to which the  $F - \log I_m$  curves for separate individuals rise, and similar differences in the slopes upon a probability grid No 7, at 21.5° (1) and at 29.5° (2),  $F_{max} = 52.75$  No 9 at 21.5° (3) and at 29.5° (4),  $F_{max} = 52.63$

magnitude of P.E.<sub>11</sub> is practically identical with that obtained with other animals<sup>1</sup>

In cases where a homogeneous lot of reacting animals is available the nature of the variation<sup>2</sup> of  $I_1$  and of  $F_1$  in the reciprocal experiments can be shown to require that the  $I_m$  and  $F_m$  curves cannot be identical For the data on *Pseudemys* this must be tested by measure



ments with the separate individuals, and these should ideally be made in pairs at the same time. The graphs in Fig 9 show that the vertical

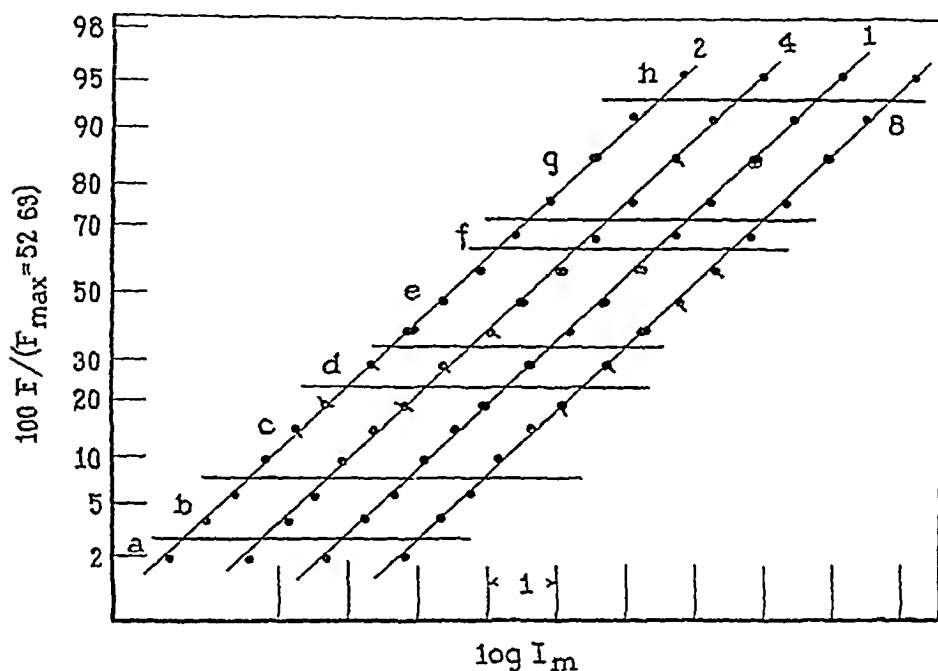


FIG 6 Data for four typical individuals (Nos 1, 2, 4, 8), giving  $\log I_1$  on a probability grid as a function of  $F$ , showing the consistency of certain minor departures from the fitted curve. Horizontal lines separate regions in which particular light-sources (lamps) and filters were employed. It is notable that there is a distinct correlation, probably traceable to the inescapable deficiencies in precision of calibration of lamps and filters.

	<i>Lamp</i>	<i>Filter</i>
<i>a</i>	100 w.	"1 10,000"
<i>b</i>	"	"1 1,000"
<i>c</i>	"	"1 100"
<i>d</i>	"	"1 10"
<i>e</i>	"	None
<i>f</i>	500 w	"
<i>g</i>	1000 w	"
<i>h</i>	2 × 1000 w	"

separation of the  $F_m$  and  $I_m$  curves is definite enough, although small. This is expected since the variation of  $F_c$  (Table II) is quite small.

(in correlation with the relatively low general slope of the  $F - \log I_m$  curve, Fig 1), the  $F_m$  and  $I_m$  observations were not made at the same time, and no determinations were made of  $F_m$  at measured values of  $I_m$ , consequently it is only in the central region of the graph that any consistent difference could be looked for (Fig 9). More numerous determinations would probably show that the mean *within individual* variation in  $F_c$  (Fig 10) would rise to a more distinct max

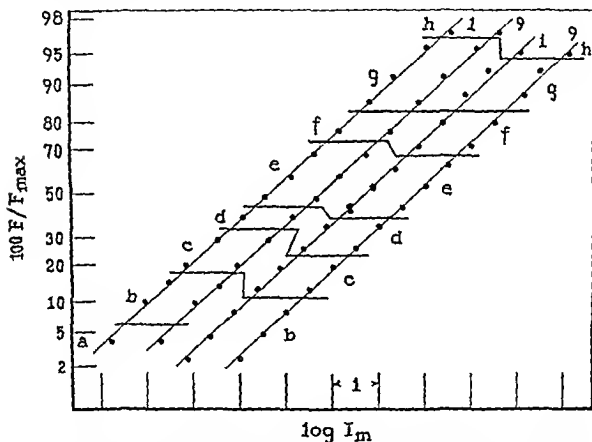


FIG 7 Data on two individuals (Nos 1 and 9) at two temperatures (21.5 and 29.5), determinations of  $F_1$  as a function of  $\log I$ . The small deviations as in Fig 6, are correlated with the use of particular lamps and filters (cf Fig 6)

imum<sup>1</sup> just beyond the inflection point of the  $F - \log I_m$  graph, this is obscured by the relatively larger proportion of instrumental variation which<sup>1</sup> becomes more important at higher levels of  $F$  and which harks larger in this case because  $P L_{1P}$  is so low

The variation of  $I_1$  is clearly *not* predominantly of instrumental or manipulative origin, but is due to differences between the individuals at the times of measurement, as the rank order analysis proves. The differences are of exactly the same order as those found with lots of

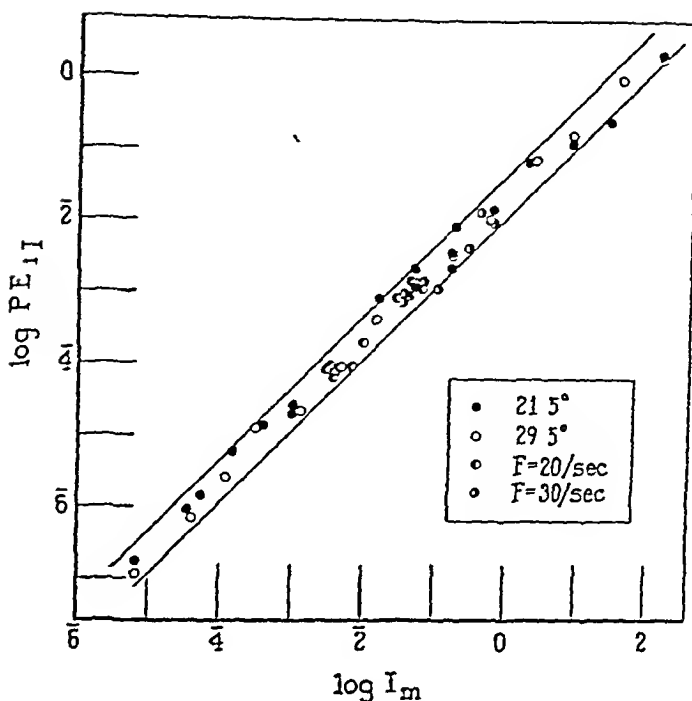


FIG 8 The dispersions of  $I_f$  at different flash frequencies for measurements at  $21.5^\circ$ ,  $29.5^\circ$ , and at various temperatures for  $F = 20$  and  $F = 30$ .  $\log P E_{11}$  is a rectilinear function of  $\log I_m$ , with a slope of 1, over the whole range

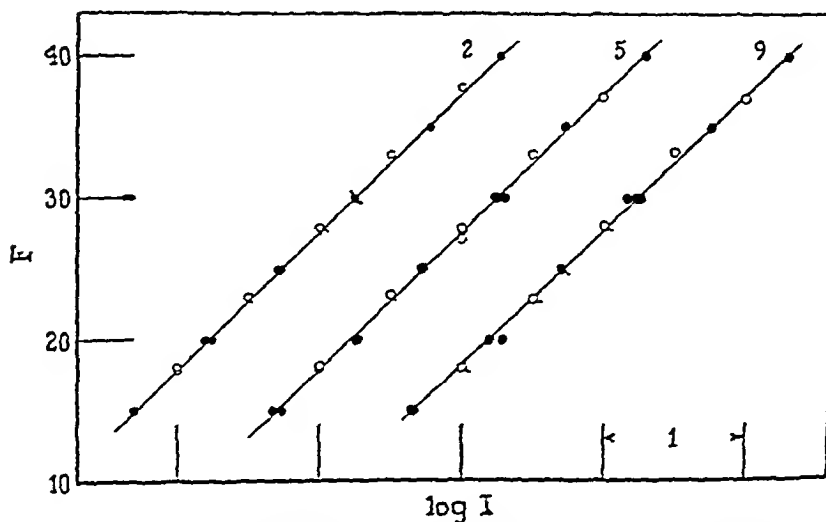


FIG 9 The relation between  $F$  and  $\log I$ , shown for individuals Nos. 2, 5, and 9, over the central part of the graph where the curve (Fig. 1) is practically rectilinear. Mean values of  $I_c$  (solid circles) definitely tend to lie below values of  $F_1$  (open circles). See text

other kinds of animals. With *Anax* these differences tend to persist<sup>9</sup> for a short time, but over longer intervals they fluctuate at random. This persistence appears in tests with different proportions of light time to dark time in the flash cycle,<sup>10</sup> but it is not found for animals with equivalent portions of the surface of the eye opaqued<sup>11</sup>—a fact for which there is a simple physical explanation,<sup>11</sup> and which justifies the view that the differences are of individual, organic nature.

With various species and types of fishes we have used the fluctuation in relative sensitivity is more rapid. In *Pseudemys* the individual differences tend to persist through long series of tests. Thus a kind of experimental verification is obtained for the opinion<sup>1</sup> that the

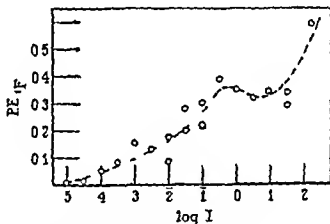


FIG 10  $P E_{1/F}$  as a function of  $\log I$  rises to a minor maximum in the region of the  $F - \log I$  inflection (cf Fig 1)

temporary differences of similar order observed with other animals (and, in man and in *Anax* for a single individual<sup>9</sup>) are likewise to be regarded as essentially organic rather than merely due to "experimental error."

#### IV

Observations on the dragon fly larva *Anax*<sup>12</sup> and on the teleost *Enneacanthus*<sup>13</sup> showed that with elevation of temperature the curve

<sup>9</sup> 1936-37 b

<sup>10</sup> 1937-38 c

<sup>11</sup> 1937-38 c

<sup>12</sup> 1936-37 c

<sup>13</sup> 1936-37 d

of  $F$  vs  $\log I_m$  was moved to lower intensities without change of shape<sup>14</sup> and with retention of the same maximum. Figs 1 and 2 demonstrate that this is true for the flicker curve of *Pseudemys* also. With *Anax* and *Enneacanthus* three temperatures were employed (12.4°, 21.5°, 27.3°), covering the workable range. While this was done mainly to detect any change of form which the curve might exhibit, it was also supposed that the complexity of the flicker recognition process might well make any simple analysis of the underlying kinetics in terms of the relation to temperature impossible. This was thought to be supported by the fact that the temperature coefficient of  $F_m$  is a function of  $I$ , while in terms of the activation energy (*temperature characteristic*)<sup>15</sup> equation the abscissa of inflection of the curve gave a non-rectilinear relationship to  $1/T_{ab}$ . The latter argument turns out to be erroneous, and, in view of our experience with many experiments bearing upon this matter,<sup>16</sup> was not very intelligent. The point, of course, is that in biological systems there are often encountered critical temperatures<sup>15</sup> with different temperature characteristics above and below this critical level,<sup>15</sup> data at three temperatures cannot, in general, be expected to be of much use in such a case if a critical temperature lies between the extremes.<sup>15</sup> The development of the theory of the flicker contour, in the meanwhile, now permits certain rational expectations to be entertained concerning the properties of more extensive measurements at a series of closely spaced temperatures.

The data are summarized in Table III. They show that the movement of the curve toward lower intensities as the temperature is increased is a regular phenomenon. The proportionate change is the same at  $F = 20$  and  $F = 30$ , and (in conjunction with the full curves determined at 21.5° and at 29.5°) allows the contention that the form of the curve, and its maximum, are independent of temperature. The data also show, in conformity with those on *Anax*<sup>11</sup> and *Enneacanthus*,<sup>13</sup> that the  $P E_{17}$  vs  $I_m$  curve is very little influenced by temperature (Fig 8), despite the marked influence upon the speed and amplitude of the index response (Section I), which is clearly consis-

<sup>14</sup> 1936-37 *d*, 1937-38 *c*

<sup>15</sup> Crozier, 1924-25 *a*, p 124, 1925-26 *b*, p 531

<sup>16</sup> *Cf* Crozier, 1924-25 *b*, 1934-35

tent with the view that the variation in  $I_1$  cannot be due to experimental error

The results of using  $I$ , temperature, and proportion of light time in a flash cycle<sup>17</sup> as variables require that  $1/I$  be taken as a measure of excitability at a given flash frequency. It has been pointed out<sup>18</sup>

TABLE III

The dispersion of the measurements of critical flash frequency ( $PE_{P_1}$ ) at the inflection point of the  $F - \log I$  graph is positively correlated with the slope of the curve at this level [ $\Delta F/(\Delta \log I \approx 1)$ ]. This is a necessary consequence of the theory that the breadth of the band defining the relationship between  $F$  and  $I$  is due to the variability of the discriminatory performance of the organism, since  $PE_{\log I}$  is constant in this region of the curve and is practically the same for all the organisms tested (cf Fig 11). The figures in parentheses refer to "rod" portions of flicker response contours

Animal	Slope $\Delta F/(\Delta \log I \approx 1)$	$PE_{P_1}$
<i>Anax</i> *	44	1 10
<i>Enneacanthus</i> †	18	0 76
(Sunfish)	(4)	(0 24)
<i>Man</i> ‡		
C	12	0 40
	(6 5)	(0 16)
W	10 4	0 30
	(7 0)	(0 10)
<i>Triturus</i> §	9	0 37
(Newt)	(4 6)	(0 10)
<i>Pseudemys</i>	6	0 17
(Turtle)		

\* Crozier, Wolf, and Zerrahn Wolf, 1936-37 b

† 1936-37 a

‡ 1937-38 b

§ 1938 d

that  $I$  has for the flicker response the significance of  $\Delta I$  in a test of intensity discrimination, and we may refer here to the fact that the corresponding use of  $1/\Delta I$  as a measure of excitability can be shown to

<sup>17</sup> 1937-38 d, c

<sup>18</sup> Crozier, 1935-36, 1936

have decided usefulness for the analysis of differential sensitivity<sup>19</sup> The effectiveness of a given  $I$ , at fixed  $F$ , will depend upon the frequency with which the excitable elements are found in a potentially excitable state There are several ways in which this frequency could be supposed to be controlled The relevant properties of the whole population of excitable elements could be governed by a uniform system of chemical events, so that with rising temperature the whole frequency distribution of excitability thresholds (in terms of  $1/I$ ) should be shifted to a higher level but not changed in form The fact that the duration of the latent period ( $LP$ ) for onset of response to light, in various forms,<sup>20</sup> is governed by the temperature in a manner described by the temperature characteristic equation  $\ln(LP) = -\mu/RT + \text{const}$  could be used as an argument for the control of the properties of an assemblage of excitable elements by the velocities of chemical events in the common medium bathing them Or it could be supposed that the internal metabolic processes common to each excitable element are influenced in the same manner by the temperature The latter hypothesis would, however, seem to require that each element would then be more frequently excitable by the flashes which succeed one another during an exposure, hence  $F_{max}$  would be expected to appear as a function of temperature, which is not observed When the proportion of light time to dark time ( $t_L/t_D$ ) in the flash cycle is decreased, it can be supposed<sup>13</sup> that the chance of finding any element in an excitable condition has been improved by the lengthening of the dark time, and in this way the observed changes in  $F_{max}$  and in the position of the flicker contour,<sup>13 12</sup> without change in its form, can be accounted for quantitatively Thus the parameters of the flicker contour are affected in a significantly different way by change of  $t_L/t_D$  and by change of temperature elevation of temperature and decrease of  $t_L/t_D$  each move the curve toward a lower intensity level, and the shift of the inflection point is directly proportional to the change in percentage light time, the slope of the curve, on a percentage ordinate ( $\sigma'_{log I}$ ) is not changed in either case, change of temperature does not affect  $F_{max}$ , but  $F_{max}$  is a rectilinear function of  $t_L/(t_L + t_D)$  Alteration of the percentage light time

<sup>19</sup> Crozier, 1936, Crozier and Holway, 1937, 1938, Holway and Crozier, 1937

<sup>20</sup> Cf Hecht, 1934

therefore influences the total number of effective available excitable elements (by making each unit more or less frequently available, but affecting all the units in the same proportion), whereas this number is not a function of the temperature. Consequently elevation of temperature does not multiply the frequency with which each element contributes to the determination of the index response, the increase of  $F$  at fixed  $I$  with rise of temperature, in the curve between the asymptotes, is merely a mechanical consequence of the fact that the position of the curve on the  $\log I$  axis has been shifted. Hence we must provisionally turn to some form of the first hypothesis, namely

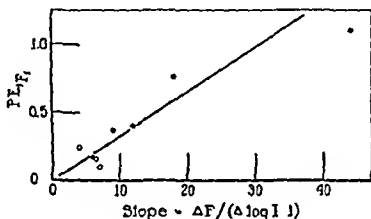


FIG 11 Data in Table III, showing the dependence of  $P E_{F,F_i}$  at homologous points (inflections) on the flicker response curve of various animals upon the slopes of the curves at these points. The open circles refer to "rod" segments of flicker contours. The departures are naturally greater at the upper end since  $P E_{F,F_i}$  is proportional to  $P E_F$ .

that the dependence of the position of the curve on the temperature is due to the effect of conditions uniformly influencing the excitability of all the elements concerned. It is to be recognized that the elements in question are defined solely in terms of the effect produced, namely the response to flicker at critical values of  $F$  and  $I$ .

If the control of excitability by temperature is similar to that apparent in many other biological processes, we should then look for the behavior of  $1/I$  to be such that the measure of excitability is proportional to the velocity of underlying chemical changes. The magnitude of the crude temperature coefficient is consistent with this view. The data are plotted in Fig 11. To a very good approxima



tion the mean values of  $1/I$  adhere to the Arrhenius equation, with  $\mu = 26\,500$  between  $F = 12.2$  and  $F = 30.0$   $\mu = 12\,400$  between  $30^\circ$  and  $35.8^\circ$ . The deviations on the intensity axis are at most =  $0.05$  log unit this is about the extreme difference to be expected in duplicate determinations of  $I_r$ . The chief reason for the departures is to be found however in the control of the temperature. In water the turtle's temperature is not noticeably higher than that of the water<sup>22</sup> but on removal from the thermostat the temperature of the aquarium changed during the 3 to 5 minutes required for an observation and changed more the greater the difference between the experimental temperature and that of the room ( $21.5^\circ$ ). This was corrected for by taking the temperature of aquaria during the period of observation, in control tests. The change at the highest and lowest points was about  $0.6^\circ$ . The mean temperatures recorded in Table III are the average temperatures during the time of observation. Probably the temperature of the turtle does not change so rapidly. In Fig. 11 the same slopes have been assigned to the lines drawn for  $F = 20$  and  $F = 30$ . These flash frequencies are situated one on either side of the inflection point of the  $F - \log I$  curve. The behavior of  $1/I_r$ , the critical excitability at the inflection point, must therefore be understood to follow the same rule.

The measurements in Table III are brought together for comparison by dividing each  $1/I_r$  at  $F = 20$  by a constant (=  $10.21$ ). The resulting figures are plotted in Fig. 12 with the curves expressing  $\ln(1/I_r) = -\mu/RT + \text{const}$ . It is clearly impracticable to pass a single curve through the whole course of the data. An adequate description is given by recognition of the critical temperature at  $29.5^\circ$ . In this region, and also at the higher temperatures as contrasted with the lower, the scatter of the determinations should be greater, as is uniformly found in such curves<sup>23</sup>. This particular critical temperature  $30^\circ \pm 0.5^\circ$  is one rather frequently found<sup>24</sup>. The behavior of  $P E_{11}$  (Table III) is such that it is directly proportional to  $I_r$   $F$  constant, when  $I_r$  is changed by altering the temperature. This means that the variation of  $(1/I)$  is a constant percentage of the

<sup>22</sup> Cf. also Isserlin, 1902.

<sup>23</sup> Crozier, 1929, 1935.

<sup>24</sup> Crozier, 1925-26 a

mean ( $1/I_m$ ) at all temperatures,—the characteristic result obtained with other biological events for which a chemical control may be assumed <sup>22</sup>

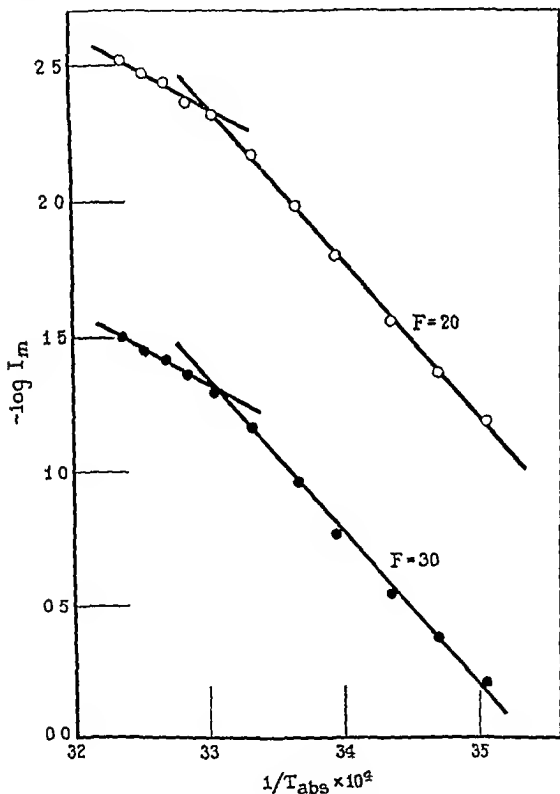


FIG 12  $\log 1/I_m$  vs  $1/T_{abs}$ , at  $F = 20$  and at  $F = 30$ , Table IV See text

In our earlier accounts of the shift of the flicker response curve with change of temperature we have utilized<sup>14</sup> the conception of the building up of an effect due to a flash and its decay in the dark interval. The effect responsible for reaction to flicker is then supposed to be due to the recognition of a mean difference between  $E_2$  (in the flash) and  $E_1$  (in the dark interval). Elevation of the temperature leads to a more rapid decay of effect in the dark, hence at given flash

TABLE IV

*Mean Critical Intensities for Response to Flicker by the Turtle Pseudemys at Flash Frequencies 20/Second and 30/Second, at Different Temperatures*

The reciprocals of the mean critical intensities (millilamberts) are given for  $F = 30/\text{second}$ , for  $F = 20/\text{second}$ , shown divided by 10 21

°C	F			
	20		30	
Corr	$\log I_m$	$1/I_m/10\ 21$	$\log I_m$	$1/I_m$
12 2	$\bar{2}\ 811$	1 51 <sub>4</sub>	$\bar{1}\ 791$	1 618
15 07	$\bar{2}\ 634$	2 28	$\bar{1}\ 624$	2 377
18 03	$\bar{2}\ 442$	3 54	$\bar{1}\ 455$	3 508
21 5	$\bar{2}\ 204$	6 12 <sub>4</sub>	$\bar{1}\ 237, \bar{1}\ 231$ ( $\bar{1}\ 266$ )	5 795
23 95	$\bar{2}\ 017$	9 42	$\bar{1}\ 039$	9 141
26 90	$\bar{3}\ 826$	14 62	$\bar{2}\ 835$	14 62
29 50		20 94	$\bar{2}\ 704$	19 77
31 32	$\bar{3}\ 635$	22 70	$\bar{2}\ 639$	22 96
32 80	$\bar{3}\ 561$	26 92	$\bar{2}\ 582$	26 18
34 30	$\bar{3}\ 530$	29 51	$\bar{2}\ 65$	28 18
35 83	$\bar{3}\ 480$	32 43	$\bar{2}\ 498$	31 77

frequency a lower intensity is required to produce a critical balance. The excitability, as we have defined it by  $1/I_m$ , increases as if under the control of the velocity of some chemical transformation in a system comprising at least two discrete processes with distinct temperature characteristics.

It is instructive to inquire how this finding can be reconciled with the intensity discrimination theory of recognition of visual flicker. Qualitatively, in terms of this hypothesis, the summated action of

the nervous units involved is pictured as producing the rising segment of the "effect" curve and the decay curve for the action of a flash,<sup>11</sup> the velocity constant of the decay curve, other things constant, determines the level at which  $I$  must arrive to be critical. At given  $I$ , more flash sensitive elements will be excited at a higher temperature, the flash effect curve will rise more steeply toward a higher maximum, presumably without change in its velocity constant, and to achieve marginal response to flicker each flash must be allowed to act for a shorter time, hence the critical  $P$  rises, until at a sufficiently high intensity all the elements available are activated. This works out graphically in the manner indicated in a previous paper.<sup>14</sup>

In the discussion of an earlier group of experiments we have pointed out that a basis can be suggested for interpreting a change in  $P/E$  at constant  $I_m$  when the temperature is altered.<sup>14</sup> The measurements with *Pseudemys* give little evidence for such a change in this case (Fig 11), but sufficiently complete sets of data are available for two temperatures only.

Taking the picture in Fig 12 as due to the fact that the excitability ( $1/I$ ) of the neural elements concerned in marginal response to flicker is governed by the velocities of chemical events common to all of the elements concerned, we have to inquire as to the kind of mechanism which could determine control by a different process on either side of a critical temperature. The general theory of such situations has been that the two temperature characteristics each refer to a specific process (reaction), both of them involved in the control of the velocity which governs the property measured (frequency of a rhythmic activity, excitability) in its relation to temperature, below the critical temperature, one of these processes is in the slow or "master" rôle, above it the other. It was pointed out long ago<sup>3</sup> that the existence of particular critical temperatures makes it impossible to assume that in general the relationship between the two processes is one of mass action succession. It is necessary to appeal to something in the nature of a phase change, or to the structure of chain reactions, in some critical location<sup>23</sup> which affects the over all speed of the process whose temperature characteristic is evidenced over the higher temperature segment.

The modification must then be usually such that the process giving rise to the  $\mu$  observed for the upper segment has had its velocity curve abruptly lowered, without change of its  $\mu$ . The occurrence of observable shifts in velocity *without* change of  $\mu$ ,<sup>24</sup> and with such changes,<sup>25</sup> is proof of the physical reasonableness of this conception, which also permits a rational interpretation of the experimental modifications of the shapes of temperature curves.<sup>16</sup> However, there is also evidence<sup>26</sup> of experimentally induced slow changes of critical temperatures in some instances, these are easily understood as due to a direct effect upon the mechanism responsible for the "phase change."

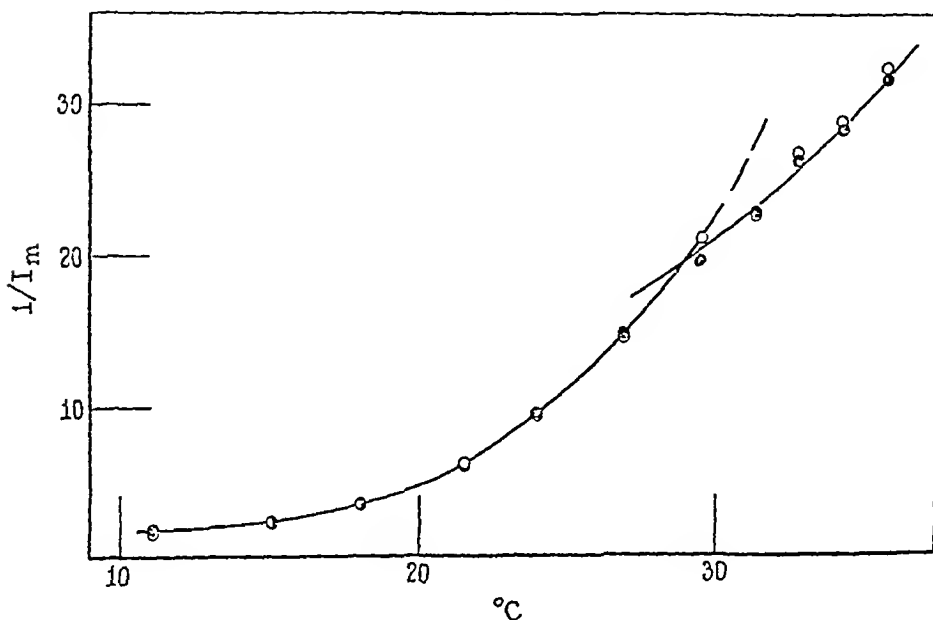


FIG 13  $1/I_m$  vs  $^{\circ}\text{C}$ , the lines are transferred from Fig 12. The data for  $F=20$  have been divided by 10.21 to bring the two sets of measurements together.

Certain of the relationships contemplated may be made clearer by a diagram (Fig 13). It is necessary, in general, to regard each of the processes revealed in a composite  $\mu$ -graph as an individualized entity, since it behaves as such in its response to experimental treatments.<sup>24,27</sup> The fact that the variation in the measured property is, over a straight section of the graph, a constant fraction

<sup>24</sup> Crozier, 1924-25 b, 1925-26 b, Crozier and Stier, 1924-25 b, Pincus, 1930-31, etc

<sup>25</sup> Crozier and Stier, 1925-26, 1926-27 a, b

<sup>26</sup> Experiments in course of publication

<sup>27</sup> Crozier and Stier, 1924-25 a c, Navez, 1936

of the mean (independent of temperature) is extremely difficult to account for if the controlling process identified by its  $\mu$ , is really a composite. Hence the two processes (Fig 12) must be conceived as linked in a chain of some kind. Both are involved in the control of the appearance of the end result which is the basis of the measurements, whichever is slowest impresses its temperature char

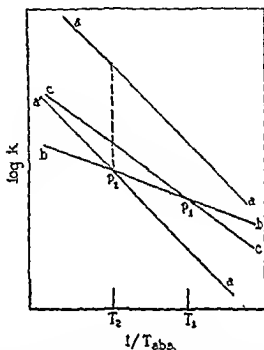


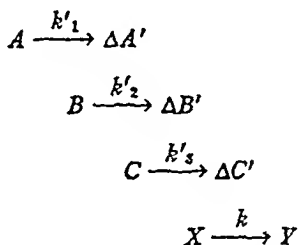
FIG 14 To indicate diagrammatically the basis for control of the magnitude of a phenomenon governed by a system of three concurrent processes catalytically linked in the manner discussed in the text. The over all frequency of the phenomenon, as a function of temperature is determined by the speed (frequency) of the process ( $a$ ,  $b$  or  $c$ ) which has the smallest  $k$ . Thus if process  $a$  were to be described by  $a - a$  in the figure, a critical temperature ( $T_1$ ) would appear at  $P_1$  and the Arrhenius plot for the observed frequency would be  $b - P_1 - c$ . Since critical temperatures markedly tend to occur at particular levels of temperature rather than in random distribution appeal must be made to shifts such as from  $a' - a'$  to  $a - a$  at a particular temperature ( $T_2$ ) if such a shift due to a phase change or analogous modification in some suitable location, produces a sufficient elevation of  $k$  without changing the slope of the Arrhenius plot the over all observed frequency will follow the broken line  $b - P_2 - a'$  shifts of the type  $a - a$  to  $a' - a'$  are known

acteristic upon the over all velocity of the whole. Physical models of this sort are easily contrived,<sup>28</sup> but are likely to be misleading. It is sufficient to suppose that we have to do with two processes which provide materials making excitation

<sup>28</sup> Cf., e.g., Hoagland, 1937

possible, the excitability will rise with the rising rates at which each of these provide their particular substances

The simplest assumption seems to be that we may consider a catenary set of catalytic processes,  $A, B, C, \dots$ , in which the rate of the terminal process, say  $C$ , determines the velocity underlying the control of the observed phenomenon. The observed phenomenon is taken to behave as if controlled by the velocity of a transformation  $X \xrightarrow{k} Y$ . The velocity constant  $k$  of this process governs the behavior of the observed phenomenon (here,  $1/I_m$ ) as a function of the temperature. The rates of the processes  $A, B$ , and  $C$  are defined in terms of the *frequencies* with which fixed quantities of their products are produced. For consistency in discussion this type of definition is necessary, since the nature of the formulation of the end result—the relation of the observed critical driving potential to temperature—depends upon the implicit definition of critical intensity as connected with the magnitude of the sensory effect which must be brought about (at a given flash frequency) this definition is obtained by means of the equation defining the dependence of  $I$  upon  $F$ , and is stated in terms of a summation of *frequencies* of elemental neural effects. The idea is specifically that, as in periodic contact catalyses, the step  $B \rightarrow B'$  cannot proceed until a quantity of material  $\Delta A'$  has formed from  $A$ , and cannot recommence until a new  $\Delta A'$  has been formed. The *frequency* with which the reaction  $B \rightarrow B'$  can begin must consequently depend upon the magnitude of  $k_1$  in the scheme



It is obvious that the frequency with which  $\Delta C'$  is produced, in a series of such steps, will depend upon the smallest value of  $k'$ . Thus the velocity constant  $k$  will be governed by (proportional to) the value of this particular  $k'$ . Nothing need be specified as to the mechanism whereby  $\Delta A'$  releases the process  $B \rightarrow B'$ , it could be by covering, or rupturing, a film on a catalytically active surface, or by other means, the transformations of  $A \rightarrow A'$ , etc., could be, and might well be, cyclic. In general, the critical increments for  $k'_1, k'_2, k'_3$ , etc., will not be the same. Hence there is opportunity for change of temperature to alter the step for which  $k'$  is the slowest. It necessarily follows from such a scheme that if a change of this kind is brought about, each  $k'$  increasing with temperature, that the particular new  $k'$  brought into control over a higher range of temperature will have a *smaller*  $\mu$  than that apparent over a lower range. Thus in Fig 13, only the  $k'$  having a smaller  $\mu$  (*i.e.*  $k'_a$ ) can possibly have its curve cross that for  $k'_a$ , assumed to be smallest at the lowest temperatures

It will be observed that this mechanism provides sharp breaks in the curve of  $k$  vs  $1/T$ , provided  $k'_1, k'_2, k'_3$  are not too far apart and have the proper disposition of  $\mu$ 's. It does not, however, explain why the breaks predominate at particular temperatures, nor why there do also occur occasionally (at these temperatures) shifts of control to a  $\mu$  or a  $k$  which is higher.<sup>29</sup> Catalysis, however, occurs at film covered surfaces, the constitution of surface films is such that critical temperatures may be a prominent property,<sup>30</sup> the doubling of a covering barrier film could easily produce a shift of a  $k'$ , with or without altering its position as smallest in a set, and thus produce a change in the over all  $k$  with or without altering its  $\mu$ , or it could, equally well, by changing one value of  $k'$  bring it (with its  $\mu$ ) into the governing, slowest position at higher temperatures.

There are other forms which this type of hypothesis can take, without affecting its fundamental nature. It allows for the introduction of effects which may (under some conditions) modify the position of a critical temperature, and it gives a basis for the interpretation of the known experimental modification of  $k$  and  $\mu$ . Moreover, it suffices to account for the fact that the relative variation of the quantity taken as proportional to  $k$  ( $\propto 1/I$ ) is independent of the temperature, in the present case, for example,  $\sigma(1/I)/1/I \approx \sigma_1/I$ , for  $T$  constant, is independent of temperature. This is easily accounted for quantitatively on the basis that the magnitude of the controlling frequency constant— $e g k_B$  or  $k_C$ , fluctuates at random.

The basic fact about the necessary guiding assumptions is that the assumed processes (reactions) are supposed to have speeds governed by the mean rates of formation of catalysts,<sup>31</sup> not by mass action of the concentrations of substrates. This elementary point has been curiously overlooked by at least one critic of the "master reaction" conception.<sup>32</sup> The kinetic equations for successive irreversible mass action processes long ago<sup>31, 33</sup> made it clear that sharp breaks in the over all rate curve could not be characteristically produced by altering the temperature, with such systems. For that reason, of course,

<sup>29</sup> Cf Pincus, 1930-31. (Cases in which the plot of  $\log k$  vs  $1/T$  is concave upward ( $\mu$  increasing continuously with  $1/T$ ) obviously signify the concurrent influence of at least two independent processes (cf Crozier 1924-25 b). An instance in which the data for a case of this sort gave rather striking confirmation of the theory of specific temperature characteristics when suitably analyzed although differently regarded by their author, is found in a recent paper by Korr (1937).)

<sup>30</sup> Langmuir, 1933.

<sup>31</sup> Crozier, 1924-25 b.

<sup>32</sup> Burton, 1936, cf Hoagland, 1937.

<sup>33</sup> Rakowski, 1907.



and for the equally good reason that the breaks occur at definite temperatures, no schemes on this basis were ever presented, and it was early pointed out<sup>23</sup> that appeal must be made to a different basis for shifts from one  $\mu$  to another

#### SUMMARY

1 At constant temperature, with a fixed proportion of light time in a flash cycle (namely,  $t_L/t_D = 1$ ), the mean critical intensity for motor response to visual flicker by the turtle *Pseudemys scripta* follows a probability integral ( $\log I$ ) as a function of flash frequency  $F$ . The fit is close and satisfactory, certain quite minor but consistent deviations are adequately explained by features of the experiments

2 The variation ( $\sigma_I$ ) of critical  $I$  is directly proportional to the mean critical intensity ( $I_m$ ), over the entire explorable range

3 These facts are consistent with the fact that the retina of this turtle is devoid of rods. It contains only cones, histologically, which, with their central representations, provide a single population of sensory effects. The properties of this population are compared with those of homologous populations deduced from corresponding measurements with other forms (various fishes, amphibian, man) which exhibit *two* such groups of sensory effects associated with the possession of retinal rods and cones

4 Certain other formulations which have previously been applied to homologous data obtained with other organisms do not properly describe the *Pseudemys* measurements

5 The use of a probability integral to describe the data of response to visual flicker for the dissection of the compound curves provided by animals possessing both rods and cones, is accordingly justified

6 Persisting differences among individuals of *Pseudemys* as regards the values of the critical flash intensity under various conditions of experimentation are of the same order of magnitude as are the transitory differences found in lots of other kinds of animals

7 Determinations of mean critical flash frequency ( $F_m$ ) at fixed levels of  $I$  lie slightly above determinations of  $I_m$  at fixed values of  $I$ , as with other forms. The variation of critical flash frequency goes through a maximum as  $\log I$  is increased, its height is lower than

with certain other forms, in correlation with the low general slope of the  $F - \log I$  curve (more properly, band)

8 These facts are consistent with the view that the dispersions of the individual critical intensities (and flash frequencies) are determined by organic variation rather than by "experimental error"

9 When the temperature is altered the  $F - \log I_m$  curve is shifted, with no change of  $F_{max}$  or of shape, the curve moves to lower intensities as the temperature is raised

10 The reciprocal of the mean critical intensity, at fixed flash frequency, is a measure of excitability With increase of temperature ( $12.5^\circ$  to  $36^\circ$ )  $1/I_m$  for given  $F$  follows the Arrhenius equation, exhibiting a "break" at  $29.5^\circ$  ( $\mu = 26,700$ ,  $12.5^\circ$  to  $29.5^\circ$ ,  $12,400$ ,  $29.5^\circ$  to  $36^\circ$ ) This is explained by the necessary theory that, the number of elements of sensory effect required for the index response at fixed  $F$  being constant, the ease of their excitation is governed by temperature through its control of the velocity of an interrelated system of catalyzed processes common to all of the sensory elements concerned<sup>24</sup>

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<sup>24</sup> Acknowledgment is made to the Elizabeth Thompson Science Fund for a grant made to one of us (W J C) for the construction of a special thermostat used in this investigation

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# THEORY AND MEASUREMENT OF VISUAL MECHANISMS

## I A VISUAL DISCRIMINOMETER II THRESHOLD STIMULUS INTENSITY AND RETINAL POSITION

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### I

#### *A Visual Discriminometer*

Various quantifiable properties of visually controlled response can be regarded as essentially expressions of a single functional process. The measurements depend fundamentally upon the performance of the organism in responding to a spatio temporal pattern of at least two physically estimated luminous intensities. According to the particular conditions imposed we may be dealing with phenomena like "intensity discrimination," "flicker fusion," "visual acuity," or the time course of sensory adaptation. In the treatment of visual excitation as a sensory phenomenon there has been a pronounced tendency to discuss such manifestations as separable and distinct. The particular visible pattern to which the organism gives an index response, or expresses discriminatory decision, is, however, the only real distinction of any one of these "visual functions."<sup>1</sup> The pattern may be one which presents two intensities simultaneously, on different parts of a visual field, or they may succeed one another in time. One of the two intensities may be equal to zero. The two intensities may be "equal," but differ as to wavelength, or as to retinal area,

<sup>1</sup> Crozier, W J, 1935-1936, *J Gen Physiol*, 19, 503 1936, *Proc Nat Acad Sc*, 22, 412

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or exposure time. The conditions may be such that time is brief and constant, or so long in special instances as to be without significance, presentation may be continuously uniform, or abruptly discrete and single, or cyclically repetitive, other parameters,—for example, retinal position,—may be involved, and in tests of different kinds each of the intensities concerned may be made to differ independently with respect to any of the other parameters. The organism itself may be permitted to vary or change,—as in a dark adaptation experiment. All of these various circumstances specifically circumscribe and delimit the conditions under which the discriminatory activity of the organism is called upon to exhibit its performance. A fundamental functional activity concerned throughout is that of *intensity discrimination*. The properties of this activity are to be defined by the manner in which its numerical measurements behave as functions of the conditions under which it is exhibited.

Certain general rules are now known to apply to the data of sensory discrimination, regardless of the particular set of conditions during a given kind of experiment. Thus if two intensities  $I_1$  and  $I_2$  are compared,  $I_1$  being fixed and  $I_2$  adjusted until just perceptibly greater than  $I_1$ , then the mean difference  $\Delta I_m = \bar{I}_2 - I_1$ , where  $\bar{I}_2$  is the mean of the adjusted values of  $I_2$ , is found in all cases to be directly proportional to the standard deviation ( $\sigma_{\Delta I_1} = \sigma_{I_2}$ ) of the distribution from which it is computed.<sup>1</sup> And the relation between  $\Delta I$  and  $I_2$  is in general of a simple and straightforward kind.<sup>2</sup> The analysis of various bodies of data shows<sup>1-3</sup> that these relationships can readily be understood from the standpoint that the performance of the organism in executing the discriminatory act is essentially one exhibiting lawful fluctuation,<sup>4</sup> the indication is that  $\sigma_{I_2}$  determines  $\Delta I_m$ , rather than the reverse.<sup>5</sup> The discriminatory performance, as measured, usually and characteristically involves the reacting organism as a whole. The immediately responsible nervous elements, however, are centrally

<sup>2</sup> Cf. Upton, M., and Crozier, W. J., 1936, *Proc. Nat. Acad. Sc.*, 22, 417.

Crozier, W. J., and Holway, A. H., 1938, *Proc. Nat. Acad. Sc.*, 24, 130.

<sup>3</sup> Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1937, *Proc. Nat. Acad. Sc.*, 23, 516. 1937-38, *J. Gen. Physiol.*, 21, 17. 1938-39, in press.

<sup>4</sup> Crozier, W. J., 1935, *Déterminisme et variabilité*, Paris, Hermann et Cie, 56 pp.

<sup>5</sup> Holway, A. H., and Crozier, W. J., 1937, *Proc. Nat. Acad. Sc.*, 23, 509.

situated, *i.e.*, are not the peripheral receptors. This makes it intelligible that the elementary rules for the data of sensory discrimination are identical (save for dimensional constants) whether one deals with photic excitation of the retina, auditory stimulation, pressure excitation, or tensive excitation of proprioceptors<sup>1</sup>. It is thus also understandable that for a given type of excitation, as by light, the *form* of the rules required is in no way dependent upon the peripheral structure of the eyes in diverse animals<sup>1, 2</sup>. Area of a retina illuminated may enter as a complex factor owing to the morphological pattern of the distribution of the sensory cells,<sup>3</sup> the gross structure of the sensory field may introduce other secondary features,<sup>4</sup> but the character of the relationship between stimulating intensity and magnitude of sensory effect may be independent of the specificities of sensory structure<sup>1</sup>.

Sensory effect is measurable only in an indirect way: its magnitudes determine the size of the intensity difference  $\Delta I$  which results from a given test of intensive discrimination. If for magnitude of sensory effect we write  $E$ , then the quantitative relation between  $E$  and  $I$  is the ultimate object of inquiry. We have to determine this relationship by means of the properties of  $\Delta I$ , to a given value of  $\Delta I_m = I_2 - I_1$  there corresponds a quantity  $\Delta E_m = E_2 - E_1$ , the problem is to determine the nature of this correspondence. The investigation thus becomes basically a study of the physiology of intensity discrimination. As such it *must* involve the physiology of the lawful variable-ness of organic performance, since this is a universal attribute of the basis of such measurements. This requires, as Helmholtz for other reasons clearly foresaw and emphasized,<sup>7</sup> a kind and a degree of care and precision in biological experimentation such as work upon the properties of non living objects *per se* rarely requires. Without this, it becomes impossible to study for its own sake the test object's contribution to the distributions of the measurements<sup>4, 8</sup>.

<sup>1</sup> Hecht, S. 1937 *Physiol. Rev.* 17, 239.

Crozier, W. J., Wolf, E., and Zerrahn, Wolf, G., 1937-38, *J. Gen. Physiol.*, 21, 223, 1938-39, in press.

<sup>2</sup> Helmholtz, H. L., 1924 *Physiological optics*, New York, Journal of the Optical Society of America. 3rd edition (translated by Southall) 2, 77.

<sup>3</sup> Crozier, W. J., 1929, *The study of living organisms* in Murchison, C., *The foundations of experimental psychology*, Worcester, Clark University Press 45.

<sup>4</sup> Sier, T. J. B., and Crozier, W. J. 1932-1933, *J. Gen. Physiol.*, 16, 757.

One source of confusion can be avoided by refraining from the practice of averaging measurements from a number of individuals. The essential requirement has been defined<sup>1</sup> as homogeneity of the data to be used. In the absence of true homogeneity, mere increase of superficial statistical precision may serve only to obscure the fundamental phenomena one is seeking to explore. With human subjects, where age and relevant genetic composition are both significant variables, the criterion can be applied, in general, only in the case of series of observations obtained with the *same* individual. A particular advantage of the human subject lies in the fact that a greater variety of tests can be made than with any given type of lower animal thus far examined. The special importance of *visual* performance (as a type of sensory response) is that a greater range of intensity (some twelve logarithmic units) can be adequately explored experimentally than with other types of sensory excitation.

The demonstration of genetically determined differences in the reactive performance of animals<sup>2, 3</sup> gives a factual, empirical basis for the abandonment of the analytical effort to learn anything about the mechanism of excitatory processes by augmenting the number of (perhaps genetically unlike) subjects to be included in a series of observations in an attempt to obtain increased precision of numerical results. With certain lower organisms this can be done, because it is possible to obtain groups of individuals which are demonstrably equivalent for the purposes of a particular experiment.<sup>3</sup> Where many human subjects have been employed by others in the past no such criterion has been applied.

A parallel and equally important requirement of the investigation of the properties of the parameters of visual excitation is that the examination of the intensity discrimination response, under diverse conditions of its exhibition, should be made with the same source of light and with the same apparatus. The significance of this requirement is obvious if one considers the problem presented by the measurement of luminous intensities in absolute units, or in units which shall be comparable if frequency (wavelength) is caused to vary. It is equally important in other ways.

<sup>2</sup> Crozier, W. J., and Pincus, G., 1929-30, *J. Gen. Physiol.*, 13, 57, 1935-36, 20, 111.

These general requirements have been especially in mind in the design of the "discriminometer" which we now describe. It was sought to devise an instrument which would permit the precise and convenient investigation of properties of human visual response such as have been traditionally grouped under such rubrics as intensity discrimination, flicker fusion, color mixture, sensory bisection, equal brilliance steps (distances), visual acuity, and the like. The requirements include the necessity of binocular as well as of monocular observations, under conditions such that the variation of critical illuminations can be investigated (and, when possible, the fluctuations in the excitability function as a whole), and the control of intensity, area, retinal location, wavelength, and duration of exposure. The work thus far done with it shows that the instrument is capable of producing highly accurate results and that it has the desired flexibility.

The optical system and a schematic plan of the visual discriminometer are shown diagrammatically in Fig 1. The general plan may be outlined first. The lenses  $L_1$  are the objectives of three collimators focussed at  $S$ , a common source of illumination. Parallel light is regularly reflected at  $90^\circ$  at  $P_1$  to the lenses  $L_2$ , which bring the rays to a focus in the plane of  $S_1$ , the bilateral slits of the discriminometer. The three uniformly illuminated apertures  $S_1$  now serve as three secondary light sources of equal intensity and essentially identical wavelength composition. Lenses  $L_3$  constitute the objectives of another triple collimator system. Parallel light enters the head of the discriminometer ( $H$ ) and then passes on through a large converging lens  $L_4$ . Light from the companion collimators passes directly into  $L_4$ , light from the other collimator is reflected  $90^\circ$  by the front surfaced mirror in the center of the head and thence into the center of  $L_4$ . This lens terminates the second series of collimators and at the same time serves as the objective lens of a binocular telescope. The inclined body of a binocular microscope head ( $MH$ ) and the eye piece  $E$  complete the general plan of the optical assembly.

The observer looking into one of the eye pieces can see a tripartite pattern, the evenly illuminated images of the apertures  $S_1$  (Fig 2). An evenly illuminated circular 'surround' (Maxwellian view) can be obtained simply and immediately by sliding any one of the lenses,  $L_3$ , so that its first focal point lies in the plane of  $L_2$ . Relative to  $S_1$  the system  $MH$  and  $L_4$  in conjunction with the lenses  $L_3$  may be regarded either as a short focus binocular telescope or as a long focus compound microscope.

The primary light source ( $S$ ) is housed in a special aluminum casting built with fins to provide a highly radiating surface. The housing is of ample size (21×21×31 cm) and can easily accommodate a 2000 watt Mazda lamp. A motor and fan tend to maintain a constant temperature by forcing a blast of cool air



through the chamber. The temperature at the cover of the lamp house, during operation, averages about  $5^{\circ}$  (or less) above that of the room. The force of the blower is regulated by a rheostat located on the base plate at the right of the discriminometer head (*cf* Fig 3). The removable top is so constructed as to permit continuous ventilation and easy access to the bulb. A stage, adjusted mechanically from outside the lamp house (vertical rack-and-pinion and plane-

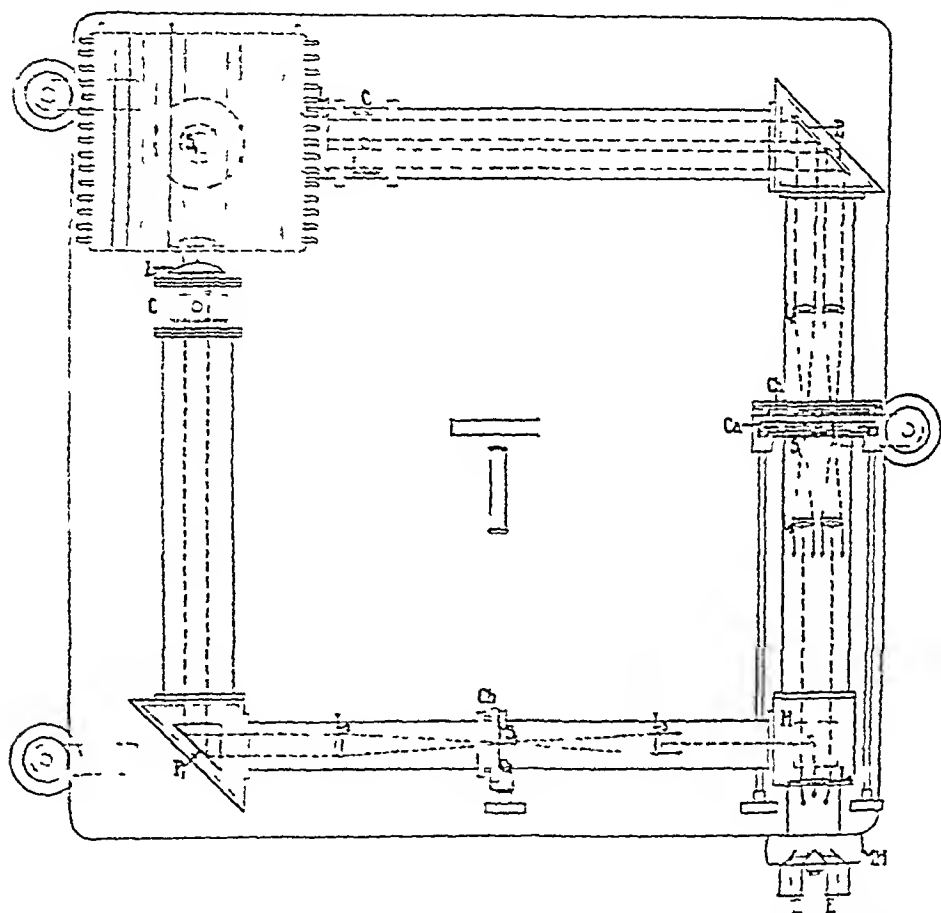


FIG 1 Schematic plan view of the visual discriminometer. For description, see text.

horizontal controls are provided), supports the lamp and insures perfect centering of the wide ribbon filament.

The light paths of the optical system are indicated by dotted lines in Fig 1. Light enters the first set of collimators and the two quartz objectives,  $L_1$ . These lenses are optically accurate and are mounted in movable ring-type holders. Cylindrical Christiansen cells ( $C$ ) are located just behind the objectives. A

rectangular isosceles prism totally reflects the light in the single collimator at  $P_1$ , while front surfaced chrom aluminized mirrors reflect the beams in the twin collimator. Each mirror is supported individually by an adjustable stage. In all cases, the reflectors are placed at  $45^\circ$  to the incident light. Upon striking these surfaces the beams are reflected at right angles into  $L_2$ , lenses that are independently mounted and appropriately stopped in cylindrical draw tubes. The draw tubes slide co axially in body tubes and can be maintained in fixed positions by means of knurled thumb screws. These adjustable lenses serve to place real, slightly magnified, and evenly illuminated images of the flat ribbon filament in the plane of each slit,  $S_1$ .

The slits are quadrilateral, each being formed between the knife-edges of two pairs of metal jaws which can be accurately and independently adjusted by means of fine pitched screws. The edges are sharp and straight. To insure paral

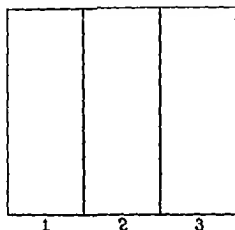


FIG 2 The form and proportions of three contiguous light patterns which can be placed on either or both retinae of the observer. The uniformly illuminated images 1, 2 and 3, can be independently varied in intensity, wavelength, size, form, time relations and retinal position: two of them, or all three can be made to overlap completely.

lelism of the slit opening the jaws are fitted exactly into two parallel guides cut in a rectangular brass frame. The frame is screwed to a bed plate of solid brass. The latter is attached to a strong support, is located in a plane perpendicular to the optical axis, and can be rotated and lifted or lowered for final alignment of the apertures in the field of view. A rectangular opening permits the passage of light through the bed plate. To minimize the chance for side play the lengths of the bearing surfaces are large relative to the size of the maximal, clear aperture. The jaws are bevelled and so placed that the flat edges face the source of light. All the elements of this assembly are uniformly black. The capstan head screws actuating the jaws work in blocks fixed to the bed plate support. In each instance the screw passes through a pair of fixed nuts, one on the bed plate the other on the slide. The screws may be easily adjusted from the outside of the instrument and can be secured in place by lock nuts. Any one

of the three slits can be increased so as to produce a clear image which subtends a visual angle of approximately  $40^\circ$  on a side at the principal point of the eye when appropriate oculars are employed

The slits  $S_1$  also lie in the focal planes of the lenses  $L_3$  which are identical with  $L_2$  with respect to mounting and adjustment. Parallel beams from  $L_3$  enter the head and then pass on to  $L_4$ .  $L_4$  is screwed into a fixed mount, and converges all of the rays passing into the head of the inclined binoculars where the rays are equally divided, twice reflected at right angles, and finally enter the paired eye-pieces,  $E$ . Coupled with the eye-pieces,  $L_4$  is thus the objective lens of a binocular telescope focussed for "infinite distance."

Each eye-piece may be supplied with a small camera lucida (Abbé type) which provides a movable fixation point in the dioptric focal plane of the observer's eye. Vertical adjustments permit the prisms of the cameras to be placed at the eye-point, and two concentric adjusting screws allow them to be independently centered. Small neutral tint filters between the eye-piece and the mirror regulate the intensity of the fixation point. Artificial pupils can be attached to the oculars. Ordinarily, however, such pupils are not necessary since the maximal diameter of the light beam at the eye-ring of our oculars is less than 2 mm.

The optical axes of the binoculars are parallel. A dial, graduated in millimeters, and conveniently located in the binocular head, is used to adjust the eye-points to the interocular distance for different observers and to reproduce settings with the same observer at different sittings. One of the ocular tubes is provided with a spiral screw to accommodate for slight differences in accommodation between the two eyes,—a very important matter for the measurement of *binocular* thresholds. A monocular head, body tube, and shield can be substituted for the binoculars when necessary. A Bausch and Lomb combination chin-rest and head-support is used to reduce extraneous movement.

All lenses are achromatic. Enclosing the body tubes of the collimators is a protective metal casing. The entire assembly is supported by vertical columns. Each of the supports is flanged at its base which rests on a smooth, heavy, cast-iron base-plate, the principle of the hole, slot, and plane is employed here and the supporting columns suffer no twisting or lateral strains. Spirit levels are mounted at right angles on the base-plate, and three levelling screws centered in circular bed-plates serve to maintain the base in a horizontal position. This accuracy in design and ruggedness in construction are necessary to insure rigidity and duration of alignment and adjustment under the severe routine of continuous use.

*Control of Intensity and Wavelength* —To control intensity, each of the light paths is supplied with appropriate mounts and holders for a wedge, a balancer, and for filters. The intensity of any one of the three beams can be varied independently (1) in stepwise fashion (neutral filters, diaphragms), or (2) continuously (wedges). The filters are clamped in small aluminum castings which fit snugly into chambers (*CH*) prepared especially to receive them. The same is true for the balancers. When necessary, filters may be placed in front of the objective lens  $L_4$  in the head of the discriminometer.

The wedges are mounted in frames and fitted securely in brass carriages (Ca) which can be raised or lowered by means of a smoothly operating helical rack and pinion adjustment. The rack is strong, the pinion gear spindles heavy. Vertical movement is imparted to any one of the carriages remotely through a steel shaft actuated by a bronze knob or hand wheel at the operating end (cf Fig 3). The knobs are conveniently accessible and easy to manipulate. Tension just sufficient to prevent slipping is exerted against the wedge carriage. The motion can be

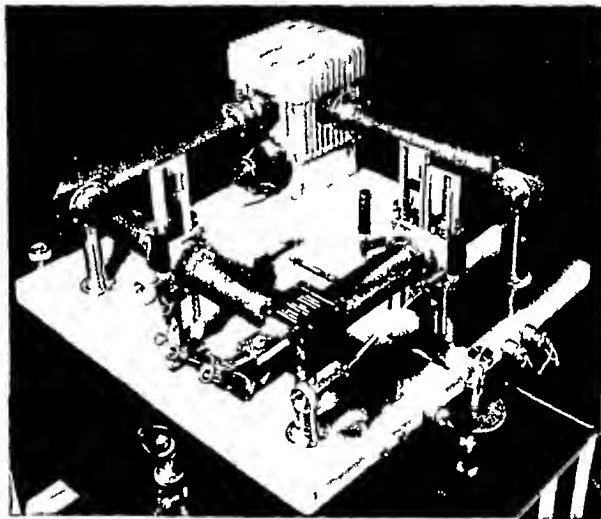


FIG 3 Perspective view of the visual discriminometer. For description see text.

stiffened or eased at will. Only slight tension is needed, however, since the area of the surfaces in contact is considerable. Universal joints remove unnecessary strain on the shafting and the surfaces are provided with oil grooves. These factors in conjunction with the working of the helical pinion gears insure smooth operation without backlash. Each carriage is supplied with a vernier scale and a shielded reading lamp. The collimator tubes are built in conveniently removable sections and large Nicol prisms can be inserted to vary intensity or for other purposes.

Intensities are measured near the eve-point either photometrically or in terms of radiant energy. The nature of the problem determines the method; this point will be discussed in subsequent reports. The range of intensities now being used extends from below the visual threshold up to a region which produces glaring and painful effects, i. e., the entire range practicable for the study of visual intensities (about twelve logarithmic units).

For wavelength control, combinations of absorbing glasses and gelatin filters can be used in the filter holders. When an unusually narrow band of wavelengths (e. g. a "line") is necessary this purity can be obtained through the addition of proper absorbing solutions placed in the quartz cells (C) and used in conjunction with an intense "monochromatic" source. A calibrated spectrometer is provided and can be attached to either of the eve-tubes for reading the wavelength of the filtered light. An image of the resulting spectrum can be placed on either or both retinæ of the observer. The intensity of this light (after calibration) can be controlled in the usual manner. Three beams of the same or of different wavelengths from a single source may thus be varied independently in intensity. By proper alignment of the apertures at the slits the images may be exactly controlled in the focal plane of either or both of the eyes to form an unusually accurate and useful color-mixer.

*Control of Size, Form, and Position of Images*—The slits  $S_1$  provide an accurate means for *continuous* variation of the area of rectangular images. However, provision is also made for apertures of fixed area of varied (e. g., circular, annular) shapes. The various shapes are produced on quartz plates, the region surrounding the desired figure being optically stopped with a silver coating. A thin optically worked cover-glass cemented over the plate eliminates entirely the necessity for removing dust particles from the edges. These patterns can be cut to any desired form and size, the slides are readily substituted in place of the adjustable slits. The light patterns produced by these forms are sharply defined, they can be moved about and secured in any desired position within the  $40^\circ$  field of view. The linear or angular dimensions of these images are measured with the aid of a divided (objective) scale and filar micrometer.

*Control of Exposure Time, Flashes, Flicker Cycle*—The sections at C are removable, and shutters placed in the openings can provide long or relatively brief exposures of any desired image. A catoptric pendulum can readily be installed for extremely short exposures (flashes). For flicker, front-silvered rotating sectors are moved into the plane of the reflector in the discriminometer head, the motor, reducing unit, and controls are held in place by a support which is independent of the discriminometer. This provides an accurate means for altering cyclically the intensity of a sharply defined image without in any way disturbing its form or position, and also for controlling the *amplitude* of the intensity cycle. An important aspect of the latter feature is that, by the use of the rectangularly disposed light beams in the apparatus, it permits the investigation of the hitherto neglected phenomena arising when two finite intensities are cyclically alternated in a systematic way.

By the substitution of a different type of binocular head, with *two objectives* focusing upon the slit images in *S*, it is possible to investigate the properties of intensity discrimination under various conditions of difference of illumination in the two eyes, and of phase differences in flash cycles for the two eyes by binocular fusion

*Résumé*—A device has been constructed which permits the investigation of different aspects of human visual excitability over a wide range of luminous intensities, and under conditions such that various significant parameters of the visual response function are under precise control. The use of a single instrument for the investigation of superficially diverse types of visual performance contributes in a significant way to the homogeneity and comparability of the observational data.

We are under obligation to Mr A D Jones and his Optical Company, of Cambridge, for excellent work on the optical parts of this instrument, and to Mr Carl Heinrich, scientific instrument maker, of Boston, for his very superior execution of the mechanical design.

## II

### *Thresholds and Retinal Position*

Our discussion of the theoretical orientation from which quantitative visual data are to be interpreted has implied a divergence from a current and rather powerfully supported view. The measurable properties of visual performance are commonly regarded as giving information about the retinal excitatory process, including its photochemistry.<sup>6</sup> The development of this conception has been basically dependent upon the results of fitting curves by which it is attempted to correlate the properties of certain equations with numerical features of different aspects of intensity discrimination. Such equations are in fact non specific. Without independent tests of the meanings of their contained constants, they are without real significance. In at least one case, these constants do not experimentally exhibit the necessary properties.<sup>10</sup> The method of fitting the photochemical equations to the data is usually highly arbitrary, and completely

<sup>10</sup> Crozier, W J, Wolf, E, and Zerrahn Wolf, G, 1936-37, *J Gen Physiol*, 20, 393, 1937-38 *a*, 21, 313, 1937-38 *b*, 463

neglects the known properties of the variation present in the measurements. The resulting descriptions, for these different reasons, cannot be unique. We shall find it necessary to return to these matters in subsequent reports.

We are concerned now with an essentially qualitative point which is of special importance in the theory of photic excitation, namely the relations, if any, between numbers and kinds of excitable *peripheral* sense cells and the judgments of intensive discrimination. These relationships, and the amounts or rates of chemical changes supposedly associated therewith, have never been theoretically clarified. They are specifically important for the view that the data of visual excitability in an immediate, proportionate way reflect the physico-chemical organization of the retina. The fact that the human retina ordinarily contains at least two classes of excitable elements, "rods" and "cones," permits a real test of certain aspects of this whole situation. Vertebrates generally which exhibit these two classes of visual cells provide visual response curves which are in two distinguishable parts or sections. This, of course, does *not* constitute proof that the peripheral two classes of primary neurons are the determining factors in this duplexity, nor does the fact that a vertebrate known to have only cones fails to show a duplex constitution of its visual response curve.<sup>11</sup>

For the human visual apparatus scotopic ("twilight") vision is conventionally considered to be associated primarily or exclusively with the excitation of the retinal rods,<sup>5</sup> and the magnitude of the absolute threshold stimulus ( $\Delta I_0$ ) for the dark adapted human eye has been taken to depend upon the excitation of a given number of rods,<sup>11</sup> as if a satisfactory basis for the interpretation of such results were to be found simply in the excitation of peripheral sense cells. An early paper by von Kries<sup>11</sup> shows that monocular  $\Delta I_0$  for a small area *decreases* with increasing distance from the fovea, whereas the curves drawn by Kleitman and Piéron through their data purport to show that  $\Delta I_0$  *increases* with increasing distance from the fovea.<sup>12</sup> Thus

<sup>11</sup> von Kries, J., 1895, *Z. Psychol. u. Physiol. Sinnesorgane*, 9, 81, 1897, 15, 327

<sup>12</sup> Kleitman, N., and Piéron, H. (1928, *Ann. Psychol.*, 29, 57) report data which at first sight seem to contradict this finding. Their results purport to give values of  $\Delta I_0$  as a function of distance of the light image from the fovea, but at least two

in the two most complete available series of measurements bearing directly upon this question the indications are apparently contradictory, although the distances concerned differ, evidence for the evaluation of this matter is presented and discussed in the present paper

### *Method and General Procedure*

Two collimators of the discriminometer were used in the experiment. The slit opening at  $S$  was reduced in size until it presented a square light image. The size of the image was adjusted throughout the experiment so that it subtended at all times a visual angle of 4.8 minutes on a side at the nodal point of the observer's eye. A tiny aperture pierced in a thin sheet of aluminum foil, mounted in the adjacent slit base plate, served as an adjustable fixation point. Both the image of the fixation point and that of the test source were located in the first focal plane of the eye piece. A Wratten filter (No. 70), inserted at  $K$  (cf. Fig. 1), was used to color the image of the fixation point. The size of the test image and the distance separating it from the fixation image were measured directly with a filar ocular micrometer. A calibrated camera shutter centered and rigidly mounted in the head of the discriminometer, was employed to control the exposure time of the test image. Single flashes of constant duration ( $= 0.20 \pm \sigma_{\Delta I} = 0.019$  second) were used throughout the experiment. For various reasons, which need not be fully gone into here, the variation of this exposure time (ca. 10 per cent) cannot be responsible for the observed behavior of the total variation in the values of the threshold intensities ( $\sigma_{\Delta I}$ ) for retinal excitation, for example there are persistent (and therefore organic) differences in the relative variation of  $\Delta I$  for different observers in various series of measurements only a portion of which are used in the present paper (Tables I, II, and III), moreover, when the exposure time is lengthened its measured variation (with this shutter) of course increases, but  $\Delta I$  declines and so does the value of  $\sigma_{\Delta I}$ . In the present series, mean  $\sigma_{\Delta I} \approx 0.097 \Delta I$ , the proportionality constant is the same<sup>1</sup> as for other procedures in which  $\Delta I$  is obtained for finite levels of  $I_1$ , so also is  $\sigma_{\sigma_{\Delta I}}$ , this emphasizes the intimate connection of  $\Delta I$  with  $\sigma_{\Delta I}$ . The shutter was calibrated with the aid of a string galvanometer to which a high speed moving film

corrections are required for their data. The first, the cosine correction, is readily made. As the eye is turned through an angle  $\theta$ , the effective area of a constant pupil aperture (and therefore the intensity of the retinal image) is reduced by a factor very nearly equal to  $\cos \theta$ , even when an immobile eye is employed the intensity is probably diminished by  $\cos \theta$  (—a correction on this basis has of course been applied to all our data). The second correction is more complex and unfortunately, cannot be estimated. It involves the state of dark adaptation and (at extreme angles) also involves the effect of a varying stop (due to the observer's eyelid and eyelashes!)



camera was attached. A sinusoidal disturbance was impressed upon the string by alternating currents from a calibrated beat-frequency oscillator (GR type 613-A),—the amplitude of the vibrations being adjusted by means of a microvolter (WE type 509-W)

Absolute threshold stimuli were measured at eighteen different retinal positions along the 0–180° meridian for each of three practised observers. The observers were dark adapted for 45 minutes before the beginning of each experiment. Starting at an intensity well below the threshold stimulus the intensity of the retinal light image was increased by small steps until the observer first reported the appearance of the light flash. The lamp color temperature was kept at 2050° K.

Ten measurements were taken at each retinal position during a single sitting. Five of the measurements at each position were made by beginning at the extreme temporal position on the retina and then proceeding inwards to and through the fovea. The remaining sets of measurements were secured in the reverse order,—by starting at the nasal aspect and continuing through the fovea until the last position in the periphery of the temporal aspect of the retina had been reached. The curves secured by these different procedures are essentially identical in form for any given observer, and for the present purpose only the averages at each position ( $N = 10$ ) for each individual observer are required (*cf* Table 1)

## RESULTS

The results are given graphically in Fig 4. This figure shows the general form of the function relating threshold stimulus intensity ( $= \Delta I_0$ , in photons) to the angular distance of the retinal test image from the fovea. For each observer, the curve falls as the distance from the center of the fovea is made greater. None of these curves is exactly symmetrical with respect to the fovea, each function possesses its own individual characteristics,—and a more thorough study (*e g* a study utilizing a closer spacing of positions on the retina) will probably reveal small but biologically real departures from the smooth curves drawn. The data basic to these curves, however, are nicely reproducible for each observer, and are entirely sufficient for our present purposes. (The magnitude of the absolute threshold intensity also declines in this characteristic manner for most wavelengths<sup>13</sup>) For the positions on the meridian tested, our results demonstrate that the threshold intensity is a declining function of the retinal distance from the fovea,—a function which definitely tends to approach a minimal value in the periphery.<sup>13</sup> This fact has general implications for any theory of vision. It is of particular importance

<sup>13</sup> Crozier, W. J., and Holway, A. H., in preparation

for the theory which attempts to account for such measurements simply in terms of retinal rod excitation<sup>14</sup>

TABLE I

Data for *monocular* threshold stimuli,  $\Delta I$ , in units of retinal illumination (photons) as a function of the angular distance of the test image from the fovea. Size of square test image = 0.08 on a side. Duration of flash = 0.20 second. Each entry is an average of ten measurements.  $\sigma_{\Delta I}$  is the root mean square deviation of a single observation. Measurements on the *left* eye of A C S H, and W J C, *right* eye of A H H.

Angular distance from fovea along 0-180 meridian	Observers					
deg	W J C		A C S H		A H H	
	log $\Delta I$	log $\sigma \Delta I$	log $\Delta I$	log $\sigma \Delta I$	log $\Delta I$	log $\sigma \Delta I$
32	1 537	2 415	1 905	2 782	1 221	2 120
29 5			1 943	2 756	1 215	2 183
27	1 528	2 334	1 981	2 788	1 215	2 204
24	1 501	2 305			1 220	2 205
21	1 522	2 661	1 941	2 851	1 149	2 003
19			1 972	2 869	1 177	2 091
16 5	1 763	2 589	1 983	2 774	1 200	2 127
13 5	1 724	2 680	0 016	1 145	1 203	2 138
9 5	1 799	2 873	0 023	1 118	1 182	2 096
6	1 878	2 759	0 124	1 216		
5	1 924	2 764	0 142	1 138	1 207	2 316
3 5	0 205	1 382	0 504	1 629	1 283	2 366
2	0 796	1 874	0 719	1 827	1 611	2 712
1					0 496	1 484
0	0 893	1 916	1 178	0 106	0 799	1 829
1					0 412	1 420
2	0 720	1 739	1 093	0 204	0 004	1 167
3 5	0 156	1 221	0 602	1 724	1 575	2 428
5	0 077	1 006			1 238	2 133
6 5	1 918	2 883	0 196	1 309		
8	1 700	2 647				
10	1 683	2 671	0 101	1 227	1 113	3 993

<sup>14</sup> Cf the conditions in von Kries' experiments (footnote 11) the exposure time seems not to have been controlled here, and any correction would be of doubtful value. At any rate a consideration of the conditions involved in von Kries' experiments shows clearly that at least a cosine correction needs to be applied to the data.

To evaluate this latter view, we may turn to a recent histological examination of the human eye (Østerberg, 1935)<sup>15</sup> This study gives the topography of the layer of rods and cones for practically the entire human retina. Thorough counts were made on very carefully prepared sections of the perfectly normal retina of a 16 year old male, the mean error for counts of rods and cones was about 1 per cent

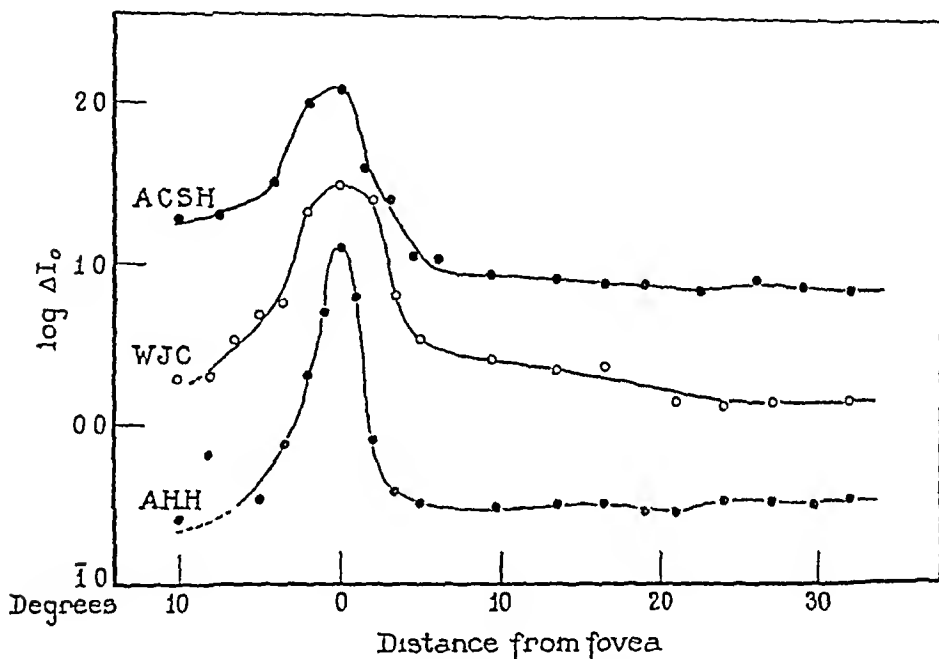


FIG 4 Absolute mean threshold stimulus intensity ( $\log \Delta I_0$ , in photons) as a function of angular distance from the fovea, for each of three observers. Size of square test image =  $0.08^\circ$  on a side. Duration of flash = 0.20 second. Each plotted point is an average of ten measurements. The ordinate scale is correct for the A H H curve, the measurements (Table I) for W J C have been increased by 0.3 log unit, for A C S H by 0.6 log unit.

(or less). The count along the  $0-180^\circ$  meridian is especially complete, and Fig 5 is a plot based on Østerberg's counts along this meridian, extending from the quartered papilla to the ora serrata in the temporal aspect. The number of primary neurons per  $\text{mm}^2$  (= density) is plotted as a function of the linear distance from the fovea

<sup>15</sup> Østerberg, G, 1935, *Acta Ophthalmol*, 13, suppl VI, 1

The figure shows clearly that the number of *cones* per mm<sup>2</sup> decreases continuously as one proceeds peripherally from the fovea. The number of *rods* per mm<sup>2</sup>, on the other hand, at first increases, passes through a maximum, and finally declines, as one proceeds from the fovea toward the periphery along the horizontal meridian. The *total number* of primary neurons (*rods plus cones*) per mm<sup>2</sup> at any point along the same meridian, computed from Østerberg's tables, is shown by the broken line. This curve necessarily decreases at first,

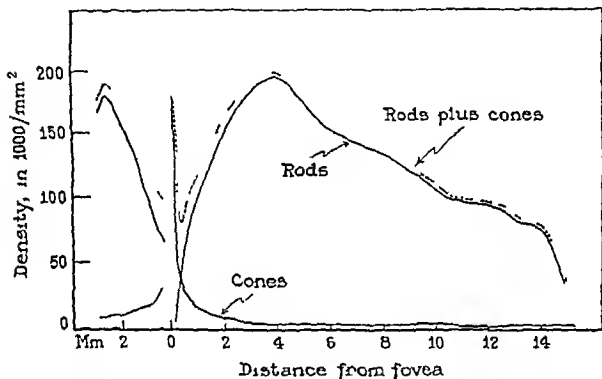


FIG 5 Plot of Østerberg's<sup>15</sup> counts of visual cells along the 0-180° meridian of the human eye

passes through a minimum, then increases, passes through a maximum, and finally declines

If the behavior of  $\Delta I$  were attributable exclusively to the rods, then under the assumptions ordinarily invoked to account for such results, the curves shown in Fig 4 should exhibit an appreciable minimum at or near a visual angle equal to 16°, and on either side of this point the curve must rise. To compare Figs 4 and 5, assume 1° = *ca* 0.29 mm. Our results manifest no such minimum. Neither do the older results of von Kries<sup>14</sup>. As a matter of fact, the curves shown

in Fig 4 tend to decline continuously even beyond a visual angle of  $30^\circ$  ( $32^\circ$  being the limit set for our measurements), or to remain at a sensibly constant level. Unless we wish to introduce additional "supporting" assumptions (*e g* an assumption to the effect that the rods increase in sensitivity beyond  $16^\circ$ ), then we are forced by the data to reject the notion that the rods alone are responsible for the magnitude of the absolute threshold stimulus for these and similar conditions of scotopic vision<sup>16</sup>. This type of argument, of course, leads immediately to a rejection of the cones *per se*, and also to the rejection of any notion like that which would simply correlate the magnitude of the visual threshold stimulus with the number of excited, primary retinal elements (rods *plus* cones)<sup>17</sup>.

The rods and cones, however, are not the only nerve cells in the human retina, and we may turn to a consideration of the secondary (collector) neurons. Although no exact counts have been made as regards the number of these "ganglion" cells per unit area, it is known that the density of these cells tends to decrease as a function of distance from the fovea<sup>18</sup>. So also does the density of optic nerve cells associated with the ganglion fibers<sup>19</sup>. The ratio of the primary nerve elements (rods plus cones) to the number of optic nerve fibers tends in general to increase as one proceeds radially from the fovea toward the periphery of the human eye. If we assume that the intrinsic excitabilities of the nerve fibers concerned are (nearly enough) equally distributed, and if the physiological process critical for the eventuation of the required effect is located in the central nervous system, we have a sufficient basis for expecting that in a general way the threshold stimulus intensity will decline as a function of distance from the fovea.

Moreover, if the occurrence of the threshold response depends upon the eventuation in the central nervous system of a particular critical effect, then we certainly have reason to expect the threshold stimulus intensity for two eyes to be *less* than that for the more sensitive eye.

<sup>16</sup> von Kries, J, 1895, *Z Psychol u Physiol Sinnesorgane*, 9, 81, 1897, 15, 327

<sup>17</sup> Wald, G, 1938, *J Gen Physiol*, 21, 269

<sup>18</sup> Poljak, S (personal communication). About one-half of these cells lie within a radius of  $6^\circ$  from the human fovea.

<sup>19</sup> Rochon-Dauvignaud, A, 1907, *Arch anat micr*, 9, 315

And this, of course, is what was found long ago by Piper<sup>20</sup> to be a fact during the course of dark adaptation. More recently<sup>21</sup>, the binocular  $\Delta I$  has been shown to be related to the average of the  $\Delta I_s$  for the left and right eyes singly by the factor 1.14. This fact at once suggests Piper's law, though the latter result was obtained for a large ( $\approx 12.5^\circ$ ) retinal image. We therefore find it of theoretical interest to test the implications of this result for a small image on different positions of the retinae.

For this purpose, light images were placed on corresponding points of the two retinae by means of the matched prism binoculars attached to the discriminometer head, and the experiment was carried out essentially as before. The barrels of the oculars were carefully adjusted until an image of *S* (cf Fig 1) was sharply defined on each retina. In securing data homogeneous in respect of the form of the function relating the magnitude of the binocular threshold stimulus to the mean angular distance from the foveae, results were secured from two dark adapted observers. Five measurements were taken at each position, beginning at an angular distance of  $15^\circ$  on the temporal aspect of the right eye and proceeding by fixed angular steps until a position equal to an angular distance of  $15^\circ$  on the temporal aspect of the left eye had been reached. The experiment was then repeated and five measurements were secured at each pair of retinal positions in the reverse order. In this way, ten measurements were obtained at every position for each mode of presentation (Table II).

Fig 6 shows the magnitude of the binocular threshold stimulus ( $\Delta I$ , in photons) as a function of the angular distance (in degrees) from the foveae along the equatorial meridian. Each observer was dark adapted 45 minutes before the measurements were taken. The measurements for each subject were secured at a single sitting, and the data are homogeneous in respect of the *form* of the function.

In general, the threshold is seen to decrease as the angular distance from the foveae is made to increase. Theoretically there need be but one exception to this rule, at about  $12^\circ$  on either side of the foveae (the region of the papilla, or "blind spot"), the threshold function

<sup>20</sup> Piper, H., 1903, *Z Psychol u Physiol Sinnesorgane* 31, 161.

<sup>21</sup> Lythgoe R. J., and Tansley L. R., 1938 *J Physiol* 91, 427. Cf also Graham, C. H. 1930 *J Gen Psychol* 3, 494.

should manifest a slight (but significant) secondary maximum. The portion of the curve lying between these limits, however, is quite symmetrical. This fact by itself is clearly consistent with the idea that the physiological effect critical for the eventuation of the threshold response is central in locus, since the monocular curves (Fig. 4) are not so symmetrical.

TABLE II

Data from each of two observers for *binocular* threshold stimulus,  $\Delta I_o$ , in units of retinal illumination (photons), as a function of the mean angular distance (degrees) of the test images from the foveae. Angular size of each square test image =  $0.08^\circ$  on a side. Duration of flash = 0.20 second. Each entry is the average of ten measurements,  $\sigma_{\Delta I_o}$  is the root-mean-square deviation of a single observation. These data are homogeneous in respect of the *form* of the function relating  $\Delta I_o$  and retinal position along the  $0-180^\circ$  meridian.

Angular distance from fovea along $0-180^\circ$ meridian	Observers			
	A C S H		A H H	
	$\log \Delta I_o$	$\log \sigma_{\Delta I_o}$	$\log \Delta I_o$	$\log \sigma_{\Delta I_o}$
15	$\bar{1} 171$	$\bar{2} 021$	$\bar{1} 053$	$\bar{3} 994$
11			$\bar{1} 037$	$\bar{3} 912$
9	$\bar{1} 245$	$\bar{2} 162$		
4 5	$\bar{1} 651$	$\bar{2} 791$	$\bar{1} 461$	$\bar{2} 224$
2	0 108	$\bar{1} 033$	$\bar{1} 988$	$\bar{2} 789$
0	0 714	$\bar{1} 614$	0 912	$\bar{1} 920$
2	0 020	$\bar{1} 125$	$\bar{1} 821$	$\bar{2} 903$
3	$\bar{1} 774$	$\bar{2} 862$		
4	$\bar{1} 599$	$\bar{2} 482$	$\bar{1} 467$	$\bar{2} 293$
6	$\bar{1} 352$	$\bar{2} 406$	$\bar{1} 273$	$\bar{2} 315$
9	$\bar{1} 127$	$\bar{2} 016$	$\bar{1} 079$	$\bar{3} 899$
12	$\bar{1} 091$	$\bar{3} 971$	$\bar{1} 061$	$\bar{2} 000$

To determine quantitatively the relationship between monocular and binocular threshold stimuli, the procedure needs to be slightly altered. If the data are to be homogeneous, then the monocular and binocular measurements must be obtained under essentially identical physiological conditions. To approximate this ideal as nearly as possible, we used Piper's technique<sup>20</sup> of staggering the monocular and binocular measurements at each set of retinal positions. As before,

the oculars were adjusted so as to reduce to a minimum the accommodation differences existing between the left and right eyes of the observer. Failure to provide for this adjustment can completely invalidate results for *binocular* thresholds. (Under commonplace conditions, an individual tends to use his more sensitive eye.) The observers were (again) dark adapted for 45 minutes. The process of dark adaptation is, of course, not complete at the close of this time

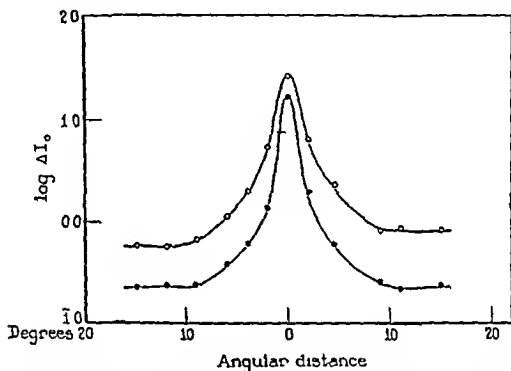


FIG 6 Magnitude of binocular threshold stimulus ( $\log \Delta I$  in photons) as a function of mean angular distance from the foveae for each of two observers. Size of square test image of each retina = 0.08 on a side. Duration of flash = 0.20 second. Each plotted point is an average of ten measurements. The data basic to the curves were obtained in such a manner as to make the functions homogeneous with respect to form. The ordinate scale applies for both curves.

interval, but its rate of change as a function of time in the dark is then slow enough to permit us conveniently to obtain reliable and strictly comparable results. At any given position, a single binocular measurement was taken first, next a monocular measurement was taken for one eye, and then one was secured for the other eye. This practice was continued systematically until finally a total of thirty measurements had been secured for each angular distance from the foveae.



The results are presented graphically in Fig 7 and numerically in Table III. The half-shaded circles represent the monocular measurements: those shaded on the left are for the left eye, those shaded on the right, for the right eye. These data are not homogeneous in respect of form, but the curves are essentially similar to those shown in

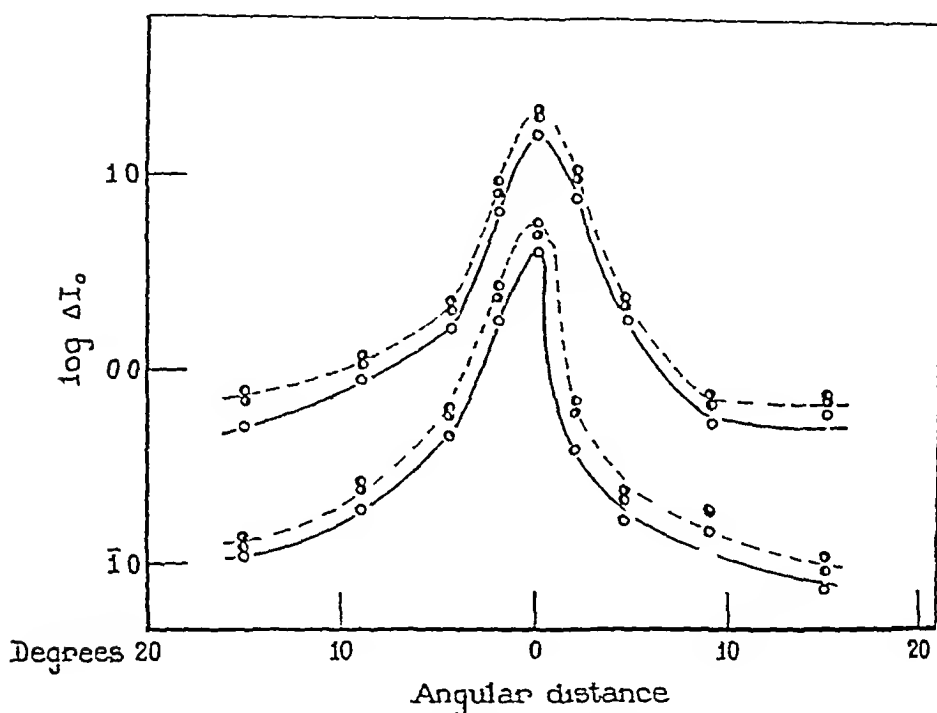


FIG 7 Homogeneous data for two observers showing that the magnitude of the binocular threshold stimulus is *less* than the magnitude of the monocular threshold stimulus for the more sensitive eye. Each point is based on ten measurements. Since two eyes give greater sensitivity than either eye *per se*, the process responsible for the threshold response may definitely be localized in the central nervous system. See text.

FIG 6 Table III shows the net result for all of the measurements as well as the measurements secured at each distance from the fovea. The binocular threshold is definitely lower than for the more sensitive eye, in each case. For each observer, the ratio of the binocular  $\Delta I_0$  to the average of the monocular  $\Delta I_0$  is, more frequently than not, and independently of retinal position, statistically equal to 1.14.

The ratio is thus nearly enough equal to  $1/\sqrt{2}$  (cf Piper's law), and hence appears to be independent of area (cf<sup>21</sup>). These results are fully coherent with a *a priori* expectation based on the assumption that the visual threshold finds its effective representation in central nervous processes. This situation may be a general one, since it applies for auditory excitation<sup>22</sup> as well, and for kinesthesia,<sup>23</sup> for

TABLE III

Data homogeneous for quantitative comparison between magnitudes of binocular and monocular threshold stimuli. Angular size of each square test image =  $0.08^\circ$  on a side. Duration of flash = 0.20 second. Each entry is an average of ten measurements,  $\sigma_{\Delta I}$  is the root mean square derivative of a single observation. See text.

Angular distance from foveae along 0-180° meridian	Observers											
	A H H						A C S H					
	Left eye		Right eye		Binocular		Left eye		Right eye		Binocular	
	log $\Delta I$	log $\sigma_{\Delta I}$	log $\Delta I$	log $\sigma_{\Delta I}$	log $\Delta I$	log $\sigma_{\Delta I}$	log $\Delta I$	log $\sigma_{\Delta I}$	log $\Delta I$	log $\sigma_{\Delta I}$	log $\Delta I$	log $\sigma_{\Delta I}$
15	1.033	3.891	1.078	3.952	2.918	3.813	1.526	2.463	1.483	2.577	1.351	2.428
9	1.364	2.294	1.325	2.464	1.217	2.192	1.671	2.785	1.719	2.738	1.592	2.616
4.5	1.732	2.666	1.707	2.871	1.594	2.389	1.950	2.927	1.993	2.944	1.863	2.845
2	0.362	1.261	0.327	1.183	0.199	1.281	0.617	1.720	0.583	1.672	0.461	1.661
0	0.682	1.573	0.643	1.582	0.540	1.410	0.982	1.936	0.989	1.937	0.830	1.829
2	1.677	2.799	1.618	2.690	1.523	2.627	0.671	1.743	0.637	1.724	0.532	1.593
4.5	1.276	2.273	1.302	2.418	1.159	2.144	0.012	1.214	1.985	2.993	1.906	2.947
9	1.206	2.163	1.205	3.998	1.100	2.035	1.479	2.493	1.530	2.622	1.377	2.520
15	2.899	3.714	2.958	3.835	2.803	3.951	1.538	2.629	1.499	2.555	1.400	2.503

audition, the ratio of the  $\Delta I$ 's for excitation of a single peripheral sensory field to those for simultaneous symmetrical excitation of bilateral fields is not, however, equal to 1.4, but is greater (= ca 2.0),

<sup>22</sup> Cf Upton, M, and Holway, A H, 1937, *Proc Nat Acad Sc*, 23, 32. We have additional data for a further analysis of this matter, in preparation.

<sup>23</sup> Holway, A H, Smith, J, and Zigler, M J, 1937, *J Exp Psychol* 20, 371.

—a fact which has considerable theoretical significance<sup>24</sup> Piper's rule<sup>25</sup> for dark adapted eyes states that  $\Delta I_0$  should be inversely proportional to  $\sqrt{A}$ , where  $A$  is the area of the test spot on a single retina. It will be noticed that we have in the present data, for fovea and for periphery, a rather precise demonstration of this rule,—but for the case in which area is doubled by applying the same area to corresponding points of two retinæ

We are under obligation to Mrs Alice C S Holway for her patient collaboration as skillful observer in these experiments

#### SUMMARY

Monocular threshold stimulus intensities ( $\Delta I_0$ , photons) were measured along the 0–180° meridian of human retinæ for three observers. The test image was small ( $= 0.08^\circ$ ) and of short duration ( $= 0.20$  second).  $\Delta I_0$  was found to decrease as the angular distance from the fovea was increased. Actual counts of the number of retinal elements per mm<sup>2</sup> along the 0–180° meridian (Østerberg<sup>15</sup>) were compared with the obtained results. No direct correlation was found to exist between visual sensitivity and the number of retinal elements.

Binocular threshold stimuli were also measured along the same meridian. The *form* of the function relating binocular visual sensitivity and retinal position was discovered to be essentially similar to that for monocular sensitivity, but is more symmetrical about the center of the fovea. The magnitude of the binocular measurement is in each case smaller than that of the monocular threshold stimulus intensity for the more sensitive eye. The ratio is statistically equal to 1.4 (a fact which suggests Piper's rule).

These results are shown to be consistent with the hypothesis that the process critical for the eventuation of the threshold response is localized in the central nervous system. They are not consistent with the view that the quantitative properties of visual data are directly determined by properties of the peripheral retina.

<sup>24</sup> We have also found, however, that over the whole workable range of intensities binocular  $\Delta I$ , as a function of  $I_1$ , is less than monocular  $\Delta I$  for either eye, but that for higher intensities the ratio is closer to 1.2 (Crozier and Holway, in preparation).

<sup>25</sup> Piper, H., 1903, *Z. Psychol u. Physiol Sinnesorgane*, **32**, 98.

# THE GROWTH OF BACTERIOPHAGE

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## INTRODUCTION

Certain large protein molecules (viruses) possess the property of multiplying within living organisms. This process, which is at once so foreign to chemistry and so fundamental to biology, is exemplified in the multiplication of bacteriophage in the presence of susceptible bacteria.

Bacteriophage offers a number of advantages for the study of the multiplication process not available with viruses which multiply at the expense of more complex hosts. It can be stored indefinitely in the absence of a host without deterioration. Its concentration can be determined with fair accuracy by several methods, and even the individual particles can be counted by d'Herelle's method. It can be concentrated, purified, and generally handled like nucleoprotein, to which class of substances it apparently belongs (Schlesinger (1) and Northrop (2)). The host organism is easy to culture and in some cases can be grown in purely synthetic media, thus the conditions of growth of the host and of the phage can be controlled and varied in a quantitative and chemically well defined way.

Before the main problem, which is elucidation of the multiplication process, can be studied, certain information regarding the behavior of phage is needed. Above all, the "natural history" of bacteriophage, i. e. its growth under a well defined set of cultural conditions, is as yet insufficiently known, the only extensive quantitative work being that of Krueger and Northrop (3) on an anti *staphylococcus* phage. The present work is a study of this problem, the growth of another phage (anti *Escherichia coli* phage) under a standardized set of culture conditions.

\* Fellow of The Rockefeller Foundation

## EXPERIMENTAL

*Bacteria Culture*—Our host organism was a strain of *Escherichia coli*, which was kindly provided by Dr C C Lindgren. Difco nutrient broth (pH 6.6–6.8) and nutrient agar were selected as culture media. These media were selected for the present work because of the complications which arise when synthetic media are used. We thus avoided the difficulties arising from the need for accessory growth factors.

*Isolation, Culture, and Storage of Phage*—A bacteriophage active against this strain of *coli* was isolated in the usual way from fresh sewage filtrates. Its homogeneity was assured by five successive single plaque isolations. The properties of this phage remained constant throughout the work. The average plaque size on 1.5 per cent agar medium was 0.5 to 1.0 mm.

Phage was prepared by adding to 25 cc of broth, 0.1 cc of a 20 hour culture of bacteria, and 0.1 cc of a previous phage preparation. After  $3\frac{1}{2}$  hours at  $37^{\circ}$  the culture had become clear, and contained about  $10^9$  phage particles.

Such lysates even though stored in the ice box, decreased in phage concentration to about 20 per cent of their initial value in 1 day, and to about 2 per cent in a week, after which they remained constant. Part of this lost phage activity was found to be present in a small quantity of a precipitate which had sedimented during this storage period.

Therefore, lysates were always filtered through Jena sintered glass filters (5 on 3 grade) immediately after preparation. The phage concentration of these filtrates also decreased on storage, though more slowly, falling to 20 per cent in a week. However, 1:100 dilutions in distilled water of the fresh filtered lysates retained a constant assay value for several months, and these diluted preparations were used in the work reported here, except where otherwise specified.

This inactivation of our undiluted filtered phage suspensions on standing is probably a result of a combination of phage and specific phage inhibiting substances from the bacteria, as suggested by Burnett (4, 5). To test this hypothesis we prepared a polysaccharide fraction from agar cultures of these bacteria, according to a method reported by Heidelberger *et al* (6). Aqueous solutions of this material, when mixed with phage suspensions, rapidly inactivated the phage.

*Method of Assay*—We have used a modification of the plaque counting method of d'Herelle (7) throughout this work for the determination of phage concentrations. Although the plaque counting method has been reported unsatisfactory by various investigators, under our conditions it has proven to be entirely satisfactory.

Phage preparations suitably diluted in 18 hour broth cultures of bacteria to give a readily countable number of plaques (100 to 1000) were spread with a bent glass capillary over the surface of nutrient agar plates which had been dried by inverting on sterile filter paper overnight. The plates were then incubated 6 to 24 hours at  $37^{\circ}\text{C}$  at which time the plaques were readily distinguishable. The 0.1 cc used for spreading was completely soaked into the agar thus prepared in

2 to 3 minutes thus giving no opportunity for the multiplication of phage in the liquid phase. Each step of each dilution was done with fresh sterile glassware. Tests of the amount of phage adhering to the glass spreaders showed that this quantity is negligible.

The time of contact between phage and bacteria in the final dilution before plating has no measurable influence on the plaque count up to 5 minutes at 25 C. Even if phage alone is spread on the plate and allowed to soak in for 10 minutes before seeding the plate with bacteria only a small decrease in plaque count is apparent (about 20 per cent). This decrease we attribute to failure of some phage particles to come into contact with bacteria.

Under parallel conditions, the reproducibility of an assay is limited by the sampling error, which in this case is equal to the square root of the number of plaques (10 per cent for counts of 100, 3.2 per cent for counts of 1000). To test the effect of phage concentration on the number of plaques obtained, successive dilutions of a phage preparation were all plated, and the number of plaques enumerated. Over a 100-fold range of dilution the plaque count was in linear proportion to the phage concentration. (See Fig. 1.)

Dreyer and Campbell Renton (8) using a different anti *coli* phage and an anti *staphylococcus* phage and a different technique found a complicated dependence of plaque count on dilution. Such a finding is incompatible with the concept that phage particles behave as single particles, i.e. without interaction, with respect to plaque formation. Our experiments showed no evidence of such a complicated behavior, and we ascribe it therefore to some secondary cause inherent in their procedure.

Bronfenhrenner and Korb (9) using a phage active against *B. dysenteriae* Shiga and a different plating technique found that when the agar concentration was changed from 1 per cent to 2.5 per cent, the number of plaques was reduced to 1 per cent of its former value. They ascribed this to a change in the water supplied to the bacteria. With the technique which we have employed variation of the agar concentration from 0.75 per cent to 3.0 per cent, had little influence on the number of plaques produced though the size decreased noticeably with increasing agar concentration. (See Table I.)

Changes in the concentration of bacteria spread with the phage on the agar plates had no important influence on the number of plaques obtained. (See Table I.) The temperature at which plates were incubated had no significant effect on the number of plaques produced. (See Table I.)

In appraising the accuracy of this method several points must be borne in mind. With our phage our experiments confirm in the main the picture proposed by d'Herelle according to which a phage particle grows in the following way: it becomes attached to a susceptible bacterium, multiplies upon or within it up to a critical time, when the newly formed phage particles are dispersed into the solution.

In the plaque counting method a single phage particle and an infected bacterium containing any number of phage particles will each give only one plaque.

This method therefore, does not give the number of phage particles but the number of loci within the solution at which one or more phage particles exist. These loci will hereafter be called "infective centers." The linear relationship between phage concentration and plaque count (Fig 1) does not prove that the number of plaques is equal to the number of infective centers, but only that it is proportional to this number. We shall call the fraction of infective centers which

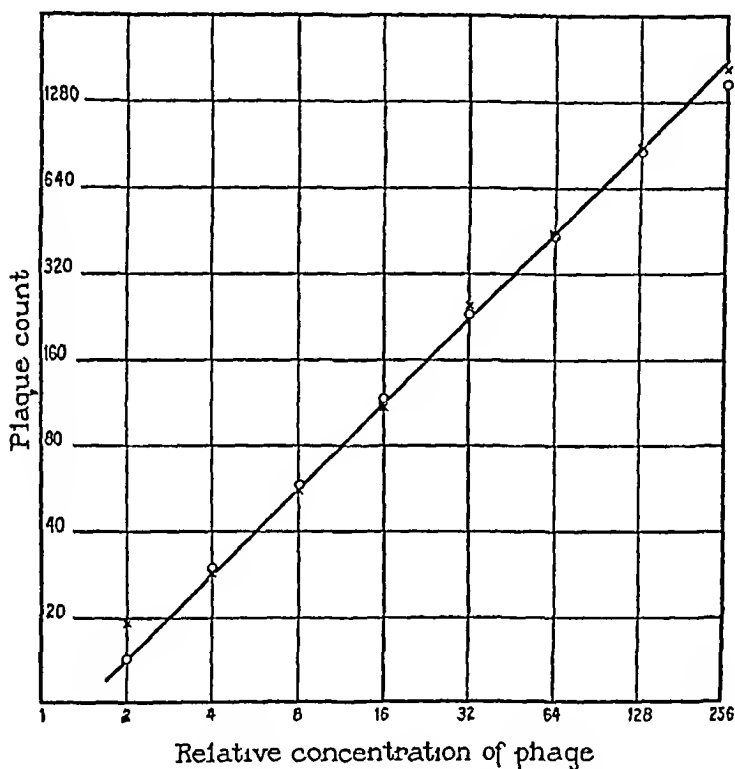


FIG 1 Proportionality of the phage concentration to the plaque count

Successive twofold dilutions of a phage preparation were plated in duplicate on nutrient agar, 0.1 cc on each plate. The plaque counts from two such series of dilutions are plotted against the relative phage concentration, both on a logarithmic scale.

produces plaques the "efficiency of plating." With the concentrations of phage and bacteria which we have used this coefficient is essentially the fraction of infected bacteria in the suspension spread on the plate, which goes through to lysis under our cultural conditions on the agar medium. After plating, the phage particles released by this lysis infect the surrounding bacteria, increasing only the size, and not the number of plaques.

TABLE I

*Independence of Plaque Count on Plating Method*

Agar concentration			
Plates were prepared in which the agar strength varied and all spread with 0.1 cc. of the same dilution of a phage preparation. There is no significant difference in the numbers of plaques obtained.			
Agar concentration, per cent	0.75	1.5	3.0
Plaque counts	394	373	424
	408	430	427
	376	443	455
	411	465	416
	373	404	469
Average	392	423	438
Plaque size, mm	2	0.5	0.2

## Concentration of plating coils

A broth suspension of bacteria ( $10^8$  bacteria/cc) was prepared from a 24 hour agar slant and used at various dilutions, as the plating suspension for a single phage dilution. There are no significant differences in the plaque counts except at the highest dilution of the bacterial suspension where the count is about 15 per cent lower.

Concentration	Plaque count
1	920
1/5	961
1/25	854
1/125	773

## Temperature of plate incubation

Twelve plates were spread with 0.1 cc of the same suspension of phage and bacteria divided into three groups and incubated at different temperatures. There were no significant differences in the plaque counts obtained.

Temperature °C	37	24	10
Plaque count	352	384	405
	343	405	377
	386	403	400
	422	479	406
Average	376	418	397



The experimental determination of the efficiency of plating is described in a later section (see p 379) The coefficient varies from 0.3 to 0.5 This means that three to five out of every ten infected bacteria produce plaques The fact that the efficiency of plating is relatively insensitive to variations in the temperature of plate incubation, density of plating *coli*, concentration of agar, etc indicates that a definite fraction of the infected bacteria in the broth cultures do not readily go through to lysis when transferred to agar plates For most experiments only the relative assay is significant, we have therefore, given the values derived directly from the plaque counts without taking into account the efficiency of plating, unless the contrary is stated

### *Growth Measurements*

The main features of the growth of this phage in broth cultures of the host are shown in Fig 2 After a small initial increase (discussed below) the number of infective centers (individual phage particles, plus infected bacteria) in the suspension remains constant for a time, then rises sharply to a new value, after which it again remains constant Later, a second sharp rise, not as clear-cut as the first, and finally a third rise occur At this time visible lysis of the bacterial suspension takes place A number of features of the growth process may be deduced from this and similar experiments, and this is the main concern of the present paper

### *The Initial Rise*

When a concentration of phage suitable for plating was added to a suspension of bacteria, and plated at once, a reproducible plaque count was obtained If the suspension with added phage was allowed to stand 5 minutes at 37°C (or 20 minutes at 25°C) the number of plaques obtained on plating the suspension was found to be 1.6 times higher This initial rise is not to be confused with the first "burst" which occurs later and increases the plaque count 70-fold After the initial rise, the new value is readily duplicated and remains constant until the start of the first burst in the growth curve (30 minutes at 37° and 60 minutes at 25°)

This initial rise we attribute not to an increase in the number of infective centers, but to an increase in the probability of plaque formation (i.e. an increase in the efficiency of plating) by infected bacteria in a progressed state, that is, bacteria in which the phage particle has commenced to multiply That this rise results from a change in the

efficiency of plating and not from a quick increase in the number of infective centers is evident from the following experiment. Bacteria were grown for 24 hours at 25°C on agar slants, then suspended in broth. Phage was added to this suspension and to a suspension of bacteria grown in the usual way, and the concentration of infective

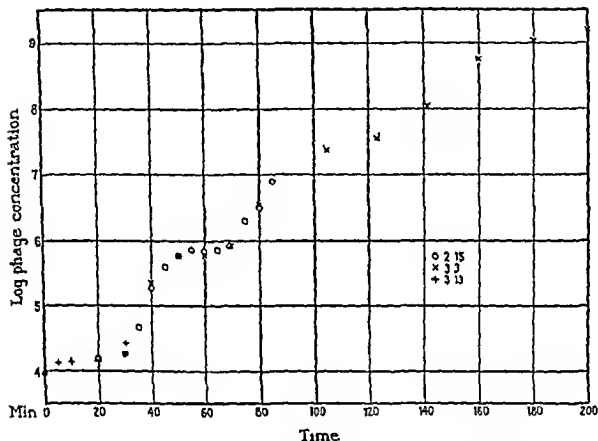


FIG. 2. Growth of phage in the presence of growing bacteria at 37°C

A diluted phage preparation was mixed with a suspension of bacteria containing  $2 \times 10^8$  organisms per cc. and diluted after 3 minutes 1 to 50 in broth. At this time about 70 per cent of the phage had become attached to bacteria. The total number of infective centers was determined at intervals on samples of this growth mixture. Three such experiments, done on different days, are plotted in this figure. The same curve was easily reproducible with all phage preparations stored under proper conditions.

centers was determined on both. The initial value was 1.6 times higher in the agar grown bacteria than in the control experiment, and remained constant until actual growth occurred. The initial rise was therefore absent in this case, clearly a result of an increase in the efficiency of plating. A sufficient number of experiments were per-

formed with bacteria grown on agar to indicate that in other respects their behavior is similar to that of the bacteria grown in broth. The bacteria grown in this way on agar slants are in some way more susceptible to lysis than the broth cultured bacteria.

### *Adsorption*

The first step in the growth of bacteriophage is its attachment to susceptible bacteria. The rate of this attachment can be readily measured by centrifuging the bacteria out of a suspension containing phage, at various times, and determining the amount of phage which remains unattached in the supernatant (*cf* Krueger (10))<sup>1</sup>

According to the picture of phage growth outlined above, phage *cannot multiply except when attached to bacteria, therefore, the rate of attachment may, under certain conditions, limit the rate of growth*. We wished to determine the rate of this adsorption so that it could be taken into account in the interpretation of growth experiments, or eliminated if possible, as a factor influencing the growth rate. Our growth curves show that there is no increase in the number of infective centers up to a critical time, we could therefore, make measurements of the adsorption on living bacteria suspended in broth, so long as the time allowed for attachment was less than the time to the start of the first burst in the growth curve. The adsorption proved to be so rapid that this time interval was ample to obtain adsorption of all but a few per cent of the free phage if the bacteria concentration was above  $3 \times 10^7$ . The number of bacteria remained constant, the lag phase in their growth was longer than the experimental period.

The rate of attachment was found to be first order with respect to the concentration of free phage ( $P_f$ ) and first order with respect to the concentration of bacteria ( $B$ ) over a wide range of concentrations, in agreement with the results reported by Krueger (10). That is, the concentration of free phage followed the equation

$$-\frac{d(P_f)}{dt} = k_a(P_f)(B)$$

<sup>1</sup>A very careful study of the adsorption of a *coli*-phage has also been made by Schlesinger (Schlesinger, M., *Z. Hyg. u. Infektionskrankh.*, 1932, 114, 136, 149). Our results, which are less accurate and complete, agree qualitatively and quantitatively with the results of his detailed studies.

in which  $k_a$  was found to be  $1.2 \times 10^{-2}$  cm<sup>3</sup>/min at 15° and  $1.9 \times 10^{-2}$  cm<sup>3</sup>/min at 25°C. These rate constants are about five times greater than those reported by Krueger (10). With our ordinary 18 hour bacteria cultures (containing  $2 \times 10^8$  *B. coli*/cc) we thus obtain 70 per cent attachment of phage in 3 minutes and 98 per cent in 10 minutes. The adsorption follows the equation accurately until more than 90 per cent attachment has been accomplished, and then slows down somewhat, indicating either that not all the phage particles have the same affinity for the bacteria, or that equilibrium is being approached. Other experiments not recorded here suggest that, if an equilibrium exists, it lies too far in favor of adsorption to be readily detected. This equation expresses the rate of adsorption even when a tenfold excess of phage over bacteria is present, indicating that a single bacterium can accommodate a large number of phage particles on its surface, as found by several previous workers (5, 10).

Krueger (10) found a true equilibrium between free and adsorbed phage. The absence of a detectable desorption in our case may result from the fixation of adsorbed phage by growth processes, since our conditions permitted growth, whereas Krueger's experiments were conducted at a temperature at which the phage could not grow.

### *Growth of Phage*

Following adsorption of the phage particle on a susceptible bacterium, multiplication occurs, though this is not apparent as an increase in the number of plaques until the bacterium releases the resulting colony of phage particles into the solution. Because the adsorption under proper conditions is so rapid and complete (as shown above) experiments could be devised in which only the influence of the processes following adsorption could be observed.

The details of these experiments were as follows. 0.1 cc of a phage suspension of appropriate concentration was added to 0.9 cc of an 18 hour broth bacterial culture, containing about  $2 \times 10^8$  *B. coli*/cc. After standing for a few minutes, 70 to 90 per cent of the phage was attached to the bacteria. At this time, the mixture was diluted 50 fold in broth (previously adjusted to the required temperature) and incubated. Samples were removed at regular intervals, and the concentration of infective centers determined.

The results of three experiments at 37°C are plotted in Fig 2, and confirm the suggestion of d'Herelle that phage multiplies under a spatial constraint, *i e* within or upon the bacterium, and is suddenly liberated in a burst. It is seen that after the initial rise (discussed above) the count of infective centers remains constant up to 30 minutes, and then rises about 70-fold above the initial value. The rise corresponds to the liberation of the phage particles which have multiplied in the initial constant period. This interpretation was verified by measurements of the free phage by centrifuging out the infected bacteria, and determining the number of phage particles in the supernatant liquid. The free phage concentration after adsorption was, of course, small compared to the total and remained constant up to the time of the first rise. It then rose steeply and became substantially equal to the total phage.

The number of bacteria lysed in this first burst is too small a fraction of the total bacteria used in these experiments to be measured as a change in turbidity, the ratio of uninfected bacteria to the total possible number of infected bacteria before the first burst is 400 to 1, the largest number of bacteria which can disappear in the first burst is therefore only 0.25 per cent of the total.

The phage particles liberated in the first burst are free to infect more bacteria. These phage particles then multiply within or on the newly infected bacteria, nevertheless, as before, the concentration of infective centers remains constant until these bacteria are lysed and release the phage which they contain into the medium. This gives the second burst which begins at about 70 minutes from the start of the experiment. Since the uninfected bacteria have been growing during this time, the bacteria lysed in the second burst amount to less than 5 per cent of the total bacteria present at this time. There is again therefore, no visible lysis.

This process is repeated, leading to a third rise of smaller magnitude starting at 120 minutes. At this time, inspection of the culture, which has until now been growing more turbid with the growth of the uninfected bacteria, shows a rapid lysis. The number of phage particles available at the end of the second rise was sufficient to infect the remainder of the bacteria.

These results are typical of a large number of such experiments, at

37°, all of which gave the 70 fold burst size, i.e. an average of 70 phage particles per infected bacterium, occurring quite accurately at the time shown, 30 minutes. Indeed, one of the most striking features of these experiments was the constancy of the time interval from adsorption to the start of the first burst. The magnitude of the rise (70 fold) was likewise readily reproducible by all phage preparations which had been stored under proper conditions to prevent deterioration (see above).

### *Multiple Infection*

The adsorption measurements showed that a single bacterium can adsorb many phage particles. The subsequent growth of phage in these "multiple infected" bacteria might conceivably lead to (a) an increase in burst size, (b) a burst at an earlier time, or (c) the same burst size at the same time, as if only one of the adsorbed particles had been effective, and the others inactivated. In the presence of very great excesses of phage, Krueger and Northrop (3) and Northrop (2) report that visible lysis of the bacteria occurs in a very short time. It was possible therefore, that in our case, the latent period could be shortened by multiple infection. To determine this point, we have made several experiments of which the following is an example. 0.8 cc. of a freshly prepared phage suspension containing  $4 \times 10^9$  particles per cc. (assay corrected for efficiency of plating) was added to 0.2 cc. of bacterial suspension containing  $4 \times 10^9$  bacteria per cc. The ratio of phage to bacteria in this mixture was 4 to 1. 5 minutes were allowed for adsorption, and then the mixture was diluted 1 to 12,500 in broth, incubated at 25°, and the growth of the phage followed by plating at 20 minute intervals, with a control growth curve in which the phage to bacteria ratio was 1 to 10. No significant difference was found either in the latent period or in the size of the burst. The bacteria which had adsorbed several phage particles behaved as if only one of these particles was effective.

### *Effect of Temperature on Latent Period and Burst Size*

A change in temperature might change either the latent period, i.e. the time of the burst, or change the size of the burst, or both. In order to obtain more accurate estimates of the burst size it is desirable

to minimize reinfection during the period of observation. This is obtained by diluting the phage-bacteria mixture (after initial contact to secure adsorption) to such an extent that the rate of adsorption then becomes extremely small. In this way, a single "cycle" of growth, (infection, growth, burst) was obtained as the following example

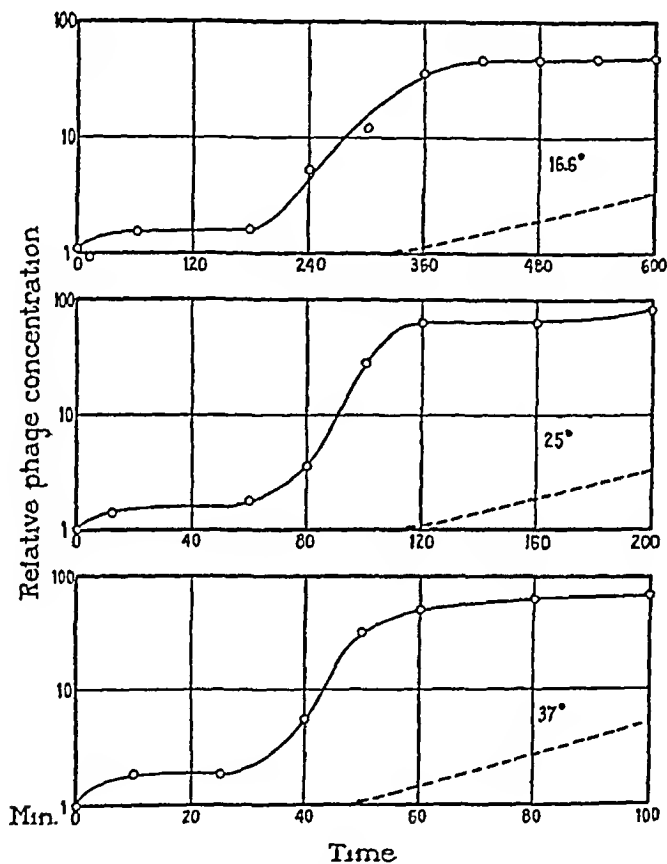


FIG 3 One-step growth curves

A suitable dilution of phage was mixed with a suspension of bacteria containing  $2 \times 10^8$  organisms per cc and allowed to stand at the indicated temperature for 10 minutes to obtain more than 90 per cent adsorption of the phage. This mixture was then diluted  $1:10^4$  in broth, and incubated. It was again diluted  $1:10$  at the start of the first rise to further decrease the rate of adsorption of the phage set free in the first step. The time scales are in the ratio 1:2:6 for the temperatures 37, 25, and 16.6°C.  $\log P/P_0$  is plotted,  $P_0$  being the initial concentration of infective centers and  $P$  the concentration at time  $t$ . The broken line indicates the growth curve of the bacteria under the corresponding conditions.

shows 0.1 cc of phage of appropriate and known concentration was added to 0.9 cc of an 18 hour culture and allowed to stand in this concentrated bacterial suspension for 10 minutes at the temperature of the experiment. This mixture was then diluted  $10^4$  in broth and incubated at the temperature chosen. Samples of this diluted mixture were withdrawn at regular intervals and assayed. The results of three such experiments are plotted in Fig. 3. The rise corresponds to the average number of phage produced per burst, and its value can be appraised better in these experiments than in the complete growth curve previously given (Fig. 2) where there is probably some overlapping of the steps. In these experiments the rise is seen to be practically identical at the three temperatures, and equals about sixty particles per infected bacterium, but the time at which the rise occurred was 30 minutes at  $37^\circ$ , 60 minutes at  $25^\circ$ , and 180 minutes at  $16.6^\circ$ . This shows that the effect of temperature is solely on the latent period.

We have also made separate measurements of the rate of bacterial growth under the conditions of these experiments. They show that the average division period of the bacteria in their logarithmic growth phase varies in the same way with temperature, as the length of the latent period of phage growth. The figures are

Temperature	Division period of <i>B</i>	Latent period of <i>P</i> growth
<i>C</i>	<i>min</i>	<i>min</i>
16.6	About 120	180
25	42	60
37	21	30

There is a constant ratio (3/2) between the latent period of phage growth and the division period of the bacteria. This coincidence suggests a connection between the time required for division of a bacterium under optimum growth conditions, and the time from its infection by phage to its lysis.

#### *Individual Phage Particle*

The growth curves described above give averages only of large numbers of bursts. They can, however, also be studied individually, as was first done by Burnett (11).



If from a mixture containing many particles very small samples are withdrawn, containing each on the average only about one or less particles, then the fraction  $p_r$  of samples containing  $r$  particles is given by Poissons' (12) formula,

$$p_r = \frac{n^r e^{-n}}{r!} \quad (1)$$

where  $n$  is the average number of particles in a sample and  $e$  is the Napierian logarithm base. If the average number  $n$  is unknown, it can be evaluated from an experimental determination of any single

TABLE II

*Distribution of Individual Particles among Small Samples*

A suitably diluted phage preparation was added to 5 cc of 18 hour bacteria culture and 0.1 cc samples of this mixture were plated. The distribution of particles among the samples is that predicted by formula (1)

	$p_r$ (experimental)	$p_r$ (calculated)
0 plaques on 13 plates	0.394	0.441
1 plaque " 14 "	0.424	0.363
2 plaques " 5 "	0.151	0.148
3 " " 1 plate	0.033	0.040
4 " " 0 plates	0.000	0.008
27 " " 33 "	1.002	1.000

one of the  $p_r$ , for instance from a determination of  $p_0$ , the fraction of samples containing no particles

$$n = -\ln p_0 \quad (2)$$

Let us now consider the following experiment. A small number of phage particles is added to a suspension containing bacteria in high concentration. Within a few minutes each phage particle has attached itself to a bacterium. The mixture is then diluted with a large volume of broth, in order to have the bacteria in low concentration so that after the first burst a long time elapses before reinfection, as in the one step growth curves. Samples (0.05 cc) are removed from this mixture to separate small vials and incubated at the desired temperature. If these samples are plated separately (after adding a drop of bacterial suspension to each vial) before the occurrence of bursts, the fraction of the plates containing 0, 1, 2, etc. plaques is found

to conform to formula (1) (see Table II) In this experiment we could also have inferred the average number of particles per sample, using formula (2), from the fraction of the plates showing no plaques (giving 0.93 per sample) instead of from the total number of plaques ( $27/33 = 0.82$  per sample)

### *Experimental Measure of Efficiency of Plating*

If the samples are incubated until the bursts have occurred, and then plated, the samples which had no particles will still show no plaques, those with one or more particles will show a large number, depending on the size of the burst, and on the efficiency of plating In any case, if we wait until all bursts have occurred, only those samples which really contained no particle will show no plaques, quite independent of any inefficiency of plating From this fraction of plates showing no plaques we can therefore evaluate the true number of particles originally present in the solution, and by comparison with the regular assay evaluate the efficiency of plating In this way we have determined our efficiency of plating to be about 0.4 For instance, one such experiment gave no plaques on 23 out of 40 plates, and many plaques on each of the remaining plates This gives  $p_0 = \frac{23}{40}$  or 0.57 from which  $n = 0.56$  particles per sample A parallel assay of the stock phage used indicated 0.22 particles per sample, the plating efficiency was therefore  $\frac{0.22}{0.56} = 0.39$  This plating efficiency remains fairly constant under our standard conditions for assay The increase in probability of plaque formation which we suppose to take place following the infection of a bacterium by the phage particle, i.e. the initial rise, brings the plating efficiency up to 0.65

### *The Burst Size*

Single particle experiments such as that described above, revealed a great fluctuation in the magnitude of individual bursts, far larger than one would expect from the differences in size of the individual bacteria in a culture, indeed, they vary from a few particles to two hundred or more Data from one such experiment are given in Table III

We at first suspected that the fluctuation in burst size was connected with the time of the burst, in that early bursts were small and late bursts big, and the fluctuation was due to the experimental superposition of these. However, measurement of a large number of bursts, plated at a time when only a small fraction of the bursts had occurred, showed the same large fluctuation. We then suspected that the particles of a burst were not liberated simultaneously, but over an interval of time. In this case one might expect a greater homogeneity

TABLE III

*Fluctuation in Individual Burst Size*

97.9 per cent of phage attached to bacteria in presence of excess bacteria (10 minutes), this mixture diluted, and samples incubated 200 minutes, then entire sample plated with added bacteria

	Bursts
25 plates show 0 plaques	
1 plate shows 1 plaque	
14 plates show bursts	130
Average burst size, taking account of probable doubles = 48	58
	26
	123
	83
	9
	31
	5
	53
	48
	72
	45
	190
	9
Total	882 plaques

in burst size, if measurements were made at a late time when they are at their maximum value. This view also was found by experiment to be false.

The cause of the great fluctuation in burst size is therefore still obscure.

## DISCUSSION

The results presented above show that the growth of this strain of phage is not uniform, but in bursts. These bursts though of constant

average size, under our conditions, vary widely in individual size. A burst occurs after a definite latent period following the adsorption of the phage on susceptible bacteria, and visible lysis coincides only with the last stepwise rise in the growth curve when the phage particles outnumber the bacteria present. It seemed reasonable to us to assume that the burst is identical with the lysis of the individual bacterium.

Krueger and Northrop (3), in their careful quantitative studies of an anti *staphylococcus* phage came to an interpretation of their results which differs in some important respects from the above.

1 Their growth curves were smooth and gave no indication of steps, they concluded therefore that the production of phage is a continuous process.

2 In their case, the free phage during the logarithmic phase of a growth curve was an almost constant small fraction of the total phage. This led them to the view that there is an equilibrium between intracellular and extracellular phage. With an improved technique, Krueger (10) found that the fraction of free phage decreased in proportion to the growth of the bacteria, in conformity with the assumption of an equilibrium between two phases.

3 Krueger and Northrop (3) found that visible lysis occurred when a critical ratio of total phage to bacteria had been attained, and they assumed that there was no lysis in the earlier period of phage growth.

To appreciate the nature of these differences it must be born in mind that their method of assay was essentially different from ours. They used, as a measure of the "activity" of the sample of phage assayed, the time required for it to lyse a test suspension of bacteria under standard conditions. This time interval, according to the picture of the growth process given here, is the composite effect of a number of factors: the average time required for adsorption of free phage, its rate of growth in the infected bacteria, the time and size of burst, and the average time required for repetition of this process until the number of phage particles exceeds the number of bacteria and infects substantially all of them. Then, after a time interval equal to the latent period, lysis occurs.

This lysis assay method tends to measure the total number of phage particles rather than the number of infective centers as the

following considerations show. Let us take a sample of a growth mixture in which is suspended one infected bacterium containing fifty phage particles. If this sample is plated, it can show but a single plaque. However, if the sample is assayed by the lysis method, this single infective center soon sets free its fifty particles (or more, if multiplication is still proceeding) and the time required to attain lysis will approximate that for fifty free particles rather than that for a single particle.

Since the burst does not lead to an increase in the number of phage particles, but only to their dispersion into the solution, the lysis method cannot give any steps in the concentration of the *total* phage in a growth curve. On the other hand one might have expected a step-wise increase in the concentration of *free* phage. However, the adsorption rate of the phage used by Krueger (10) is so slow that the infection of the bacteria is spread over a time longer than the presumed latent period, and therefore the bursts would be similarly spread in time, smoothing out any steps which might otherwise appear. Moreover, their measurements were made at 30 minute intervals, which even in our case would have been insufficient to reveal the steps.

The ratio between intracellular and extracellular phage would be determined, according to this picture of phage growth, by the ratio of the average time of adsorption to the average latent period. The average time of adsorption would decrease as the bacteria increased, shifting the ratio of intracellular to extracellular phage in precisely the manner described by Krueger (10).

As we have indicated in the description of our growth curves, lysis of bacteria should become visible only at a late time. Infection of a large fraction of the bacteria is possible only after the free phage has attained a value comparable to the number of bacteria, and visible lysis should then set in after the lapse of a latent period. At this time the total phage (by activity assay) will be already large compared to the number of bacteria, in agreement with Krueger and Northrop's findings.

It appears therefore that while Krueger and Northrop's picture does not apply to our phage and bacteria, their results do not exclude for their phage the picture which we have adopted. It would be of fundamental importance if two phages behave in such a markedly different way.

## SUMMARY

1 An anti *Escherichia coli* phage has been isolated and its behavior studied

2 A plaque counting method for this phage is described, and shown to give a number of plaques which is proportional to the phage concentration. The number of plaques is shown to be independent of agar concentration, temperature of plate incubation, and concentration of the suspension of plating bacteria

3 The efficiency of plating, i.e. the probability of plaque formation by a phage particle, depends somewhat on the culture of bacteria used for plating, and averages around 0.4

4 Methods are described to avoid the inactivation of phage by substances in the fresh lysates

5 The growth of phage can be divided into three periods: adsorption of the phage on the bacterium, growth upon or within the bacterium (latent period), and the release of the phage (burst)

6 The rate of adsorption of phage was found to be proportional to the concentration of phage and to the concentration of bacteria. The rate constant  $k_a$  is  $1.2 \times 10^{-9}$  cm<sup>3</sup>/min at 15°C and  $1.9 \times 10^{-9}$  cm<sup>3</sup>/min at 25°C

7 The average latent period varies with the temperature in the same way as the division period of the bacteria

8 The latent period before a burst of individual infected bacteria varies under constant conditions between a minimal value and about twice this value

9 The average latent period and the average burst size are neither increased nor decreased by a fourfold infection of the bacteria with phage

10 The average burst size is independent of the temperature, and is about 60 phage particles per bacterium

11 The individual bursts vary in size from a few particles to about 200. The same variability is found when the early bursts are measured separately, and when all the bursts are measured at a late time

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# THE ANTAGONISM BETWEEN SODIUM AND MAGNESIUM IONS IN THEIR ACTION UPON OXALATE ION

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In 1928 Simms (1928 *a* and *b*) described some experiments upon the anomalous effect of  $MgCl_2$  upon the dissociation constants of some organic acids. Subsequently (1928 *c*), he reported the results of the addition of sodium and potassium salts upon mixtures containing  $MgCl_2$  and oxalate buffers. He believed that his results showed an antagonism between Na (or K) ion and Mg ion similar to the well known physiological antagonism between Na and Ca ions.

It has since been shown (Cannan and Kibrick (1938), Davies (1938), Greenwald (1938 *a*), Kilde (1936), MacDougall and Larson (1937), Money and Davies (1932)) that the salts of many organic acids with calcium and other divalent metals are only partially dissociated in solution. Examination of Simms' data for the action of  $MgCl$  upon sodium oxalate, malonate, and aminoacetate showed that the effect observed is quite readily explicable in terms of the hypothesis of the formation of slightly dissociated complexes (Greenwald (1938 *b*)).

It seemed that those results that appeared to show an antagonism of ions might be due merely to the effect of the added NaCl in increasing the ionic strength of the solution and thus increasing the dissociation of the complex.

In this paper, we intend to discuss only the results with mixtures containing oxalate,  $MgCl_2$ , and NaCl. We are neglecting the results obtained with sulfate (Simms (1928 *c*)) because it is impossible to calculate the extent of formation of undissociated  $MgSO_4$ . The experiments with KCl are similarly neglected because only low concentrations of oxalate and Mg were employed, with only small effects upon the pH, and, also, because the dissociation constants of oxalic acid were determined in NaCl solution and may well be different in



KCl solution (See Harned and Robinson (1928) and Harned and Owen (1930) for similar effects in the case of acetic and formic acids)

As was shown in a previous paper (Greenwald (1938 *b*)), the formation of an undissociated complex must result in a decrease in the pH, so that to bring an oxalate buffer solution containing  $\text{MgCl}_2$  to the same pH as one of the same ionic strength, but containing no  $\text{Mg}^{++}$  or other divalent cation, would require more base. The following relation obtains

$$B' = b' (1 - \text{MgOx}) + 2\text{MgOx} \quad (1)$$

in which  $B'$  = corrected equivalents of base at given pH in presence of  $\text{MgCl}_2$

$b'$  = corrected equivalents of base at given pH in absence of  $\text{MgCl}_2$  but same ionic strength

$\text{MgOx}$  = equivalents of undissociated complex

Therefore,

$$\text{MgOx} = \frac{B' - b'}{2 - b'} \quad (2)$$

$B'$  was taken from Simms' data and  $b'$  was calculated from the equation

$$b' = \frac{(\text{H}^+) K_1}{(\text{H}^+)^2 + (\text{H}^+) K_1 + K_1 K_2} + \frac{2K_1 K_2}{(\text{H}^+)^2 + (\text{H}^+) K_1 + K_1 K_2}$$

$K_1$  and  $K_2$  were taken, directly or by extrapolation, from the data given by Simms. The ionic strengths were then recalculated, using the multiplier, 3.6, introduced by Simms (1928 *a*) for the oxalate ion. If the formation of the complex had resulted in an appreciable change in ionic strength, the calculation was repeated, using the values given by Simms for  $K_1$  and  $K_2$  at the new ionic strengths.

In solutions of low pH, as Cannan and Kibrick have shown, it is necessary to take into account the formation of  $\text{MgOxH}^+$ . They calculated the value of the negative logarithm of the hydrogen dissociation of this complex to be approximately 1.8, at  $\mu = 0.2$  or  $\sqrt{\mu} = 0.45$ . Since both the numerator and the denominator of the formula 
$$\frac{(\text{H}^+) (\text{MgOx})}{(\text{MgOxH}^+)}$$
 contain the concentration of a univalent cation, the

value of  $K$  is not likely to be greatly affected by a change in ionic strength. Assuming that  $pK$  is approximately 1.8 even up to  $\mu = 1.0$ , we may write  $pH = pK + \log \frac{(MgOx)}{(MgOxH^+)}$  or  $pH - pK = \log \frac{(MgOx)}{(MgOxH^+)}$  from which the value of  $(MgOxH^+)$ , in terms of  $(MgOx)$ , may readily be calculated.

TABLE I

Values for  $K = \frac{(Mg^{++})(Ox^-)}{(MgOx)} \cdot 10^3$  Calculated from Simms' Table III Oxalate  
Concentration = 0.01026 M

Mg = 0.00417 M				
pH	1	2†	3‡	$\sqrt{\mu}$
3.746	3.5	3.0		0.154
3.730	4.7	4.2		0.194
3.686	3.9	3.5		0.222
3.681	5.8	5.5		0.276
3.587	5.2	4.7		0.388
3.534	8.1			0.526
3.433	8.4			0.726
3.379	11.8			0.882
3.323	24.1			1.015
Mg = 0.0167 M				
3.284	3.5	3.3		0.232
3.248	11.8			0.748
Mg = 0.0417 M				
2.972	4.3	4.0	3.6	0.353
2.947	13.5		12.0	1.06
Mg = 0.0833 M				
2.764	5.1	4.9	3.9	0.495
2.808	10.8	11.2	9.6	0.866
2.776	11.4		8.9	1.00

\* First approximation

† After correcting for change in  $\sqrt{\mu}$

‡ After correcting for formation of  $MgOxH^+$

Substituting the value for  $(\text{MgOxH}^+)$  thus found in the equation

$$B' = b' (1 - \text{MgOx} - \text{MgOxH}^+) + 2\text{MgOx} + \text{MgOxH}^+ \quad (3)$$

we may calculate a revised value for  $\text{MgOx}$ . This was done in all the selected cases in which the observed pH was below 3.0, that is, in those in which the amount of  $\text{MgOxH}^+$  was at least 1/13th of that of  $\text{MgOx}$ .

TABLE II

Values for  $K = \frac{(\text{Mg}^{++})(\text{Ox}^{--})}{(\text{MgOx})} \cdot 10^3$  Calculated from Simms' Table V    Oxalate  
Concentration = 0.00513 M

Mg = 0.00167 M			
pH	1*	2†	$\sqrt{\mu}$
3.852	1.5	1.1	0.105
3.906	3.7	2.7	0.120
3.901	4.1	2.9	0.131
3.895	4.1		0.142
3.883	4.1		0.150
3.824	3.3		0.193
3.808	6.7		0.250
3.790	6.6		0.296
3.752	6.7		0.336
Mg = 0.00333 M			
3.801	3.6	1.9	0.121
3.717	6.7		0.302
Mg = 0.00833 M			
3.485	2.7	2.3	0.165
3.531	7.9		0.359

\* As in Table I

† As in Table I

It seemed unnecessary to make the calculations for all of Simms' data. Those chosen from his Tables III and V include the lowest and the highest total ionic concentration for each concentration of  $\text{MgCl}_2$  and, in addition, all of the experiments with the smallest concentrations of  $\text{MgCl}_2$  but with increasing amount of  $\text{NaCl}$ . The values obtained are summarized in the tables. The first column gives

the pH for purpose of identification. Succeeding columns give successive approximations to the value of  $K = \frac{[Mg^{++}][C_2O_4^{--}]}{[MgOx]}$  and the final column gives the revised value of  $\sqrt{\mu}$ .

It will be seen that the value of  $K$  increases quite regularly with increasing ionic strength and that this change occurs to the same extent in  $MgCl_2$  solution as in  $NaCl$ . With the highest concentration of  $MgCl_2$ , the addition of  $NaCl$  seems to have less effect upon the value of  $K$  than it does when less  $MgCl_2$  is present. Thus, at  $\mu = 1.00$  the value of  $K$  is 24.1, when the total  $Mg$  is 4.18 millimolar, 12.0 when it is 41.7, and 8.9 when it is 83.3. However, it must not be forgotten that the calculation in the first instance is based upon a very small difference between  $B'$  and  $b'$ , only 0.034 m eq.

In this instance, small changes in the value of the dissociation constants of oxalic acid have a great effect upon the values of  $B'-b'$  and of  $K$ . For instance, decreasing the negative logarithm of the first dissociation constant by 0.02 and increasing that of the second by 0.02, would change  $B'-b'$  to 0.048 m eq. and decrease  $K$  to  $15.5 \times 10^{-3}$ . A similar change applied to the data obtained with the mixture containing 83.3 m eq.  $MgCl_2$ , would change the value of the first approximation to  $K$  only slightly, from  $11.4 \times 10^{-3}$  to  $10.8 \times 10^{-3}$ .

Extrapolation of the figures given in the tables indicates that the value of the dissociation constant for  $MgOx$  at  $\mu = 0$  is, approximately,  $8 \times 10^{-4}$ . This is only slightly greater than the value,  $3.7 \times 10^{-4}$ , calculated by Monev and Davies (1932) from conductivity data.

#### SUMMARY

The action of  $NaCl$  upon the effect of  $MgCl_2$  upon oxalate buffer systems, interpreted by Simms as an instance of antagonism of  $Na^+$  and  $Mg^{++}$ , has been shown to be capable of formulation as the effect of increasing ionic strength upon the dissociation of  $MgC_2O_4$  into magnesium and oxalate ions.

<sup>1</sup> Even in Simms' tables the values reported for  $pK_T(pA)$  of oxalic acid at the same ionic strength occasionally differ by more than 0.01. For  $\sqrt{\mu} = 0.265$  the values entered in different tables are 3.886, 3.883, 3.859, 3.877.

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# ON THE DISTRIBUTION OF VITAMINS A<sub>1</sub> AND A<sub>2</sub>

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In the retinas of mammals, frogs, and certain marine fishes vitamin A participates with rhodopsin, a rose colored photosensitive pigment of the rods, in a cycle of the form  $\text{rhodopsin} \xrightleftharpoons{\text{light}} \text{retinene} \text{---protein} \longrightarrow \text{vitamin A} \text{---protein} \longrightarrow \text{rhodopsin}$  (Wald, 1935-36, 1936-37, 1937-38)

In the rods of certain fresh water fishes rhodopsin is replaced by a purple photolabile pigment which I have suggested be called *porphyropsin*. This was first observed *in situ* by Kühne and Sewall (1880), and was examined spectrographically in solution by Kottgen and Abelsdorff (1896). Porphyropsin enters a retinal cycle which is identical in form with that of rhodopsin, but contains different carotenoids in the positions of retinene and vitamin A. The substance which replaces vitamin A in the fresh water fish retina reacts with antimony chloride to yield a deep blue color due to an absorption band at about 696 mμ, accompanied by a broad subsidiary hump at about 640 mμ (Wald, 1937).

Almost simultaneously with the announcement of these observations, Lederer and Rosanova (1937) reported that substances yielding

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antimony chloride bands at about 690 and 645  $m\mu$  may predominate in the liver oils of certain Russian fresh-water fishes. This observation was rapidly confirmed and extended (Edisbury, Morton, Simpkins, and Lovern, 1938, Gillam, Heilbron, Jones, and Lederer, 1938, Lederer and Rathmann, 1938). The substance responsible for the antimony chloride band at 690–697  $m\mu$  was found to possess a direct absorption band in ethanol at 345–350  $m\mu$ . It is apparently a homologue of vitamin A, possessing one added ethylenic group and the empirical formula  $C_{22}H_{31}OH$  (Gillam *et al*). A pike-perch liver oil—which I conclude from the ratio of extinctions at 693 and at 620  $m\mu$  contained little or no vitamin A—possessed growth-promoting activity in rats about equal to that of a halibut liver oil of the same Lovibond blue value (Gillam *et al*).

Since in fresh-water fishes the 696  $m\mu$  chromogen replaces vitamin A in its most specific physiological function, the synthesis of visual purple, Edisbury *et al* (1937) have suggested that it be called vitamin A<sub>2</sub>.<sup>1</sup> The retinene analogue in these fishes may similarly be called retinene<sub>2</sub>. The porphyropsin cycle may then be formulated

$$\text{Porphyropsin} \xrightleftharpoons{\text{light}} \text{retinene}_2 \text{ --- } \rightarrow \text{vitamin A}_2 \text{ --- } \rightarrow \text{porphyropsin}$$

The substances heretofore called vitamin A and retinene are found exclusively in almost all vertebrates. Ordinarily it should be un-

<sup>1</sup> In a later paper Edisbury *et al* (1938) refer to this substance as factor A<sub>2</sub>, apparently because "there is no evidence that its function as a vitamin extends beyond fishes." More recently Gillam *et al* (1938) and Lederer and Rathmann (1938) appear to base the vitamin designation for A<sub>2</sub> principally on the demonstration of its growth-promoting activity in rats. There is some confusion in this viewpoint. The implication that the term vitamin is to be reserved to "higher" animals is equivocal, since it is already known that, for example, ascorbic acid is a vitamin in some mammals, like the guinea pig and man, and not in others, like the rat. Furthermore there is as yet no evidence that in rats A<sub>2</sub> acts directly, and not simply as a pro-vitamin A<sub>1</sub>, like a number of other carotenoids. One may choose between two consistent alternatives: to restrict the term vitamin to the human accessory factors, or to apply it to all dietary accessory factors, recognizing its limitation to those animals in which its activity has been demonstrated. I believe the latter alternative to conform most closely with present usage. In this sense the 696  $m\mu$  chromogen is vitamin A<sub>2</sub> because it replaces vitamin A<sub>1</sub> in the visual systems of certain fishes.

ambiguous to continue to refer to them without subscripts as in the past. Throughout the remainder of the present paper, however, the burden of which is a continual comparison of these substances with vitamin A<sub>2</sub> and retinene<sub>2</sub>, I shall for greater explicitness refer to the former as vitamin A<sub>1</sub> and retinene<sub>1</sub> (*cf* Lederer and Rathmann, 1938).

Band maxima of the components of the rhodopsin and porphyropsin systems and of the antimony chloride reactions with their carotenoid members are listed in Table I.

No other case is known in which a vitamin appears to be restricted to a specific group of vertebrates. It is important therefore that the precise distribution of vitamins A<sub>1</sub> and A<sub>2</sub> among fishes and its sig-

TABLE I

Absorption maxima of solutions of rhodopsin and porphyropsin in aqueous digitonin, of solutions of vitamins A and retinenes in chloroform, and of antimony chloride reactions with the latter substances in chloroform

Substance	Absorption maximum	Antimony chloride maximum
	mμ	mμ
Rhodopsin	500	—
Retinene <sub>1</sub>	387	664
Vitamin A <sub>1</sub>	328	615-620
Porphyropsin	522	—
Retinene <sub>2</sub>	405	705
Vitamin A <sub>2</sub>	355	696

nificance be determined. This is the purpose of the present experiments.

### *Plan of the Research*

Extracts have been examined from livers and from three eye tissues—the retina proper, the pigmented epithelium which is histologically and functionally closely bound with the neural retina, and the vascular choroid. All of these tissues but the retina proper may contain concentrations of vitamins A and other carotenoids which seem to exceed greatly their immediate needs, and probably represent temporarily inert stored material.

This is not in general true of the retina. Almost all retinal vitamin A<sub>1</sub> or A<sub>2</sub> is physiologically active, participating directly in the visual



cycles Practically all of it is consumed in the synthesis of visual purples and reappears following their exposure to light Retinal vitamins A therefore possess a special significance, they are diagnostic of particular types of visual purple system The retinal distribution of vitamins A ordinarily is paralleled precisely by distributions of the other components of the visual cycles, the retinenes, and rhodopsin and porphyropsin

The observations which follow are restricted to antimony chloride reactions with the vitamins A alone for purely technical reasons (1) the vitamins A are the only visual components common to all the tissues examined, and (2) the direct spectra of mixtures of rhodopsin and porphyropsin, of the retinenes, or of vitamins A, as shown in Table I, are separated by only about 20  $m\mu$ , in mixtures of these pigments the bands fuse to form single maxima in intermediate positions Even the antimony chloride bands of the retinenes, 41  $m\mu$  apart are imperfectly separated in mixtures Only the antimony chloride maxima of the vitamins A, which are separated by 76 to 81  $m\mu$ , remain distinct in mixtures of all proportions, and permit approximate estimation of their composition by simple inspection

The fishes which have been examined fall into four classes

A *Stenohaline* fishes, restricted permanently to a narrow range of salinities

- 1 Permanently fresh-water fishes
- 2 Permanently marine fishes

B *Euryhaline* fishes, capable of adult existence within a wide range of salinities

- 1 Fresh-water spawners (*anadromous*)
- 2 Marine spawners (*catadromous*)

This classification possesses certain unusual features It has been common practice to emphasize the *migratory* habits of certain fishes, and the terms anadromous and catadromous literally indicate migration from salt to fresh water or in the reverse direction However, it probably is true that no fish must depart from its spawning environment to complete a normal existence Migration to or from the sea is merely a potentiality realized in varying degree by all euryhaline fishes The fundamental characteristic is the euryhalinity of certain adult fishes, and the added fact that this is usually combined with a very high specificity of spawning environment

### *Methods*

The chemical procedures employed in these experiments have intentionally been kept extremely simple to avoid possible changes during preparation of tissues or extracts and to facilitate eventual extension of this survey to include many more animals. Unless otherwise noted, the experiments were performed in the following manner.

Retinas were dissected in the dark in Ringer's solution immediately after each individual fish had been heheaded. They were freed from all apparent traces of pigmented epithelium and choroid and were then exposed to bright light and left in moderate light at room temperatures for at least 1 hour to allow all retinenes formed on irradiation to be converted to vitamins A. The tissues were centrifuged, all excess fluid decanted, and were shaken vigorously by machine for about 20 minutes with benzine (petroleum ether, boiling range 30 to 60 C). The extraction mixture was centrifuged, and the clear benzine extract decanted and evaporated to dryness under reduced pressure. The only residue was dissolved in a small volume (usually 0.3 cc.) of dry chloroform.

After the retinas had been prepared the choroids or combined choroids and pigmented epithelia were scooped out of the sclerae into Ringer's solution and were collected by centrifuging. They were extracted by shaking with either chloroform or benzine. The extract was centrifuged clear and brought over into usually 0.3 cc. of chloroform.

Minced liver tissue was partly dehydrated by rinsing once with 95 per cent ethanol then was digested at about 70 C. in 6 per cent potassium hydroxide in methanol. Usually after about an hour the entire tissue had gone into solution. The saponification mixture was diluted with water in stages until an equal volume had been added and at each stage of dilution was extracted by shaking with benzine. The total benzine extract was washed repeatedly with water, and was distilled dry under reduced pressure. The only residue was taken up in chloroform.

When not being actively worked all preparations were stored in darkness on solid carbon dioxide.

Antimony trichloride reagent was prepared by saturating dry chloroform with fresh crystals at about 60 C. and allowing the solution to cool slowly to room temperature. Excess antimony chloride crystallizes on the sides of the container and keeps the stock solution constantly saturated at room temperatures.

Spectra were measured with the recording photoelectric spectrophotometer of Hardy (1935) at the Color Measurements Laboratory of the Massachusetts Institute of Technology. This instrument draws absorption spectra directly on graph paper either in terms of the fraction of incident light absorbed ( $1 - I/I_0$ ) or of the extinction or optical density,  $\log I_0/I$  in which  $I_0$  is the incident and  $I$  the transmitted intensity. Fig. 8 is an original recording, all the other figures have been traced on tracing cloth from similar records. In a few instances the originals have previously been published (Wald 1936-37).

The spectrophotometric procedure was rigidly standardized. 0.3 cc. of tissue

extract in chloroform was poured into a glass cell of 3.6 cc capacity and 10 mm depth. This was placed in position in the spectrophotometer. 3.2 cc of antimony chloride reagent were rapidly introduced by pipette, and recording of the spectrum began immediately. The wavelength interval from 580 to 720  $m\mu$  was negotiated in about 1 minute. During this period the initial blue test color fades slightly, but the distortion of spectrum introduced in this way is very small.

*Estimation of Vitamins  $A_1$  and  $A_2$  in Mixtures* — Edisbury *et al.* and Gillam *et al.* have tacitly assumed that the antimony chloride bands of vitamins  $A_1$  and  $A_2$  do not overlap, and that their concentrations in mixtures are therefore represented directly by the extinctions at about 620 and 693  $m\mu$  respectively. Actually the bands do overlap considerably, and this way of estimating concentrations is consequently seriously misleading. It yields incorrect quantitative results, and implies the presence of both vitamins A in many oils which really contain one of them alone. I have used the following procedure for evaluating the proportions of vitamins  $A_1$  and  $A_2$  in mixtures.<sup>2</sup>

The antimony chloride bands of a number of vitamin  $A_1$  preparations which contained no  $A_2$ , measured in the course of the present experiments, have possessed extinctions at 696  $m\mu$   $0.08 \pm 0.03$  of those at 618  $m\mu$ . Similarly, vitamin  $A_2$  preparations which contained no  $A_1$  have yielded bands possessing extinctions at 618  $m\mu$   $0.39 \pm 0.04$  of those at 696  $m\mu$ .

If the extinction due to vitamin  $A_1$  at 618  $m\mu$  be  $x$ , then the extinction due to this vitamin at 696  $m\mu$  is  $0.08x$ . Similarly, if the extinction due to vitamin  $A_2$  at 696  $m\mu$  is  $y$ , then that at 618  $m\mu$  due to this vitamin is  $0.39y$ .

$$\left. \begin{aligned} \text{Observed total extinction at 618 } m\mu &= k_{618} = x + 0.39y \\ \text{Observed total extinction at 696 } m\mu &= k_{696} = y + 0.08x \end{aligned} \right\} \quad (1)$$

This pair of simultaneous equations reduces to the convenient form

$$\left. \begin{aligned} x &= \frac{2.56k_{618} - k_{696}}{2.48} \\ y &= k_{696} - 0.08x \end{aligned} \right\} \quad (2)$$

The extinction at 618  $m\mu$  due to vitamin  $A_1$  ( $x$ ) may be converted to absolute or a variety of arbitrary units by the use of appropriate factors available in the literature. Similar factors for dealing with vitamin  $A_2$ , however, have not yet been determined. I have therefore expressed the proportion of vitamins  $A_1$  and  $A_2$  in mixtures arbitrarily by the ratio of the extinction at 618  $m\mu$  due to vitamin  $A_1$  to that at 696  $m\mu$  due to  $A_2$  ( $x/y$ ). To make such ratios conveniently comparable, as in Table II, their components have been made to sum to 100. To state this procedure somewhat differently, the computed extinctions  $x$  and  $y$  may be

<sup>2</sup> Recently Lederer and Rathmann (1938) have discussed and offered an approximate solution for this problem.

thought of as the respective "color equivalents" of vitamins  $A_1$  and  $A_2$ . The sum of these extinctions may be taken as an arbitrary measure of the total vitamin A content, and the contribution to it of each vitamin expressed as a percentage of this total. An example follows.

A chinook salmon liver oil yielded an antimony chloride spectrum with maxima at 618 and 696  $m\mu$ , and extinctions at these wavelengths of 0.428 and 0.155 respectively. Through substitution of these values in equations (2) one obtains  $x = 0.379$  and  $y = 0.125$ . The sum of these extinctions is 0.504, of which  $0.379/0.504 = 75$  per cent is due to vitamin  $A_1$  and the remaining 25 per cent to  $A_2$ .

The 618/696  $m\mu$  extinction ratios for vitamins  $A_1$  and  $A_2$  on which this computation is based are strictly valid only for the test and spectrophotometric procedures here employed. However, Gillam *et al.* have reported 620/697 ratios of 0.46 and 0.48 for concentrates of fresh water fish liver oils, and 0.38 as their lowest such ratio. Lederer and Rathmann (1938) find a 620/695 ratio of 0.33 for their richest vitamin  $A_2$  concentrates. It is clear therefore that the experimental techniques of these workers lead to factors comparable with that of  $0.39 \pm 0.04$  used in the present experiments.

### Observations

#### A. Retinas

Spectra of the antimony chloride reactions with retinal extracts from four marine teleosts are shown in Fig. 1. Those from the black sea bass, porgy, and sea robin have previously been presented as part of a study of the visual purple systems in these animals (Wald, 1936-37). Data from the sand flounder were obtained from fish killed at sea in the light adapted condition several hours before dissection of the retinas and extraction.

These fish, representing four different families, are all permanently marine forms. It is clear from Fig. 1 that their retinas contain the 615-620  $m\mu$  chromogen, vitamin  $A_1$ , and not a trace of vitamin  $A_2$ .

Taxonomic notes on these fishes follow.

Black sea bass, *Centropristis striatus*. Family Serranidae the basses. In the same family are the striped bass, which enters brackish or fresh water to spawn, and the anadromous white perch, data from which are presented below.

Porgy or scup, *Stenotomus chrysops*. Family Sparidae the sea breams, all typically marine.

Sea robin *Prionotus carolinus*. Family Triglidae the gurnards, all marine.

Sand flounder *Lophopsetta maculata*. Family Pleuronectidae, the flounders, all marine. This species is the closest North American relative of the European turbot and haddock.

Similar data from the retinas of two marine elasmobranchs, the smooth and spiny dogfishes, are shown in Fig 2 (a, c) As in the

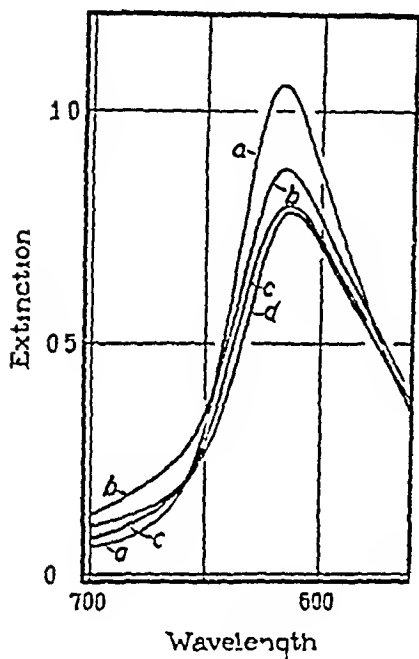


FIG 1

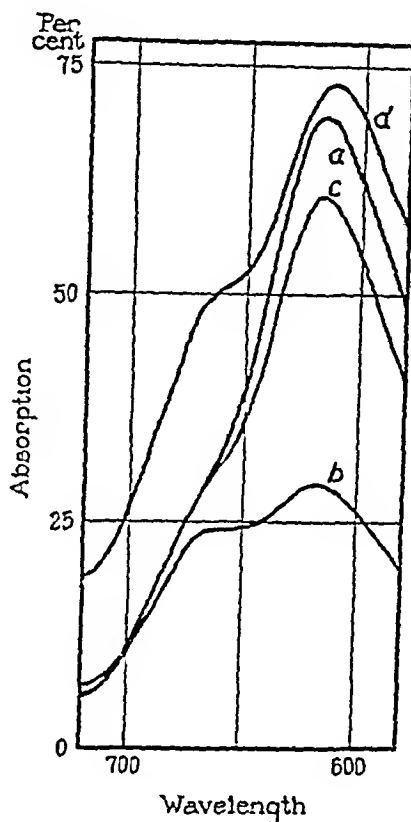


FIG 2

FIG 1 Marine teleost retinas Spectra of the antimony chloride reaction with total benzene extracts of (a) two sea bass, (b) five scup, (c) two sand flounder, and (d) five sea robin retinas These tissues contain the 618  $m\mu$  chromogen vitamin A<sub>1</sub> alone

FIG 2 Marine elasmobranch eye tissues Antimony chloride reaction with total benzene extracts of (a) four retinas and (b) eight choroids of smooth dogfish, and (c) six retinas, and (d) twelve choroids of spiny dogfish These tissues contain only vitamin A<sub>1</sub>, accompanied by small quantities of the 664  $m\mu$  chromogen, retinene<sub>1</sub>

marine teleost retinas, only vitamin A<sub>1</sub> is present, accompanied in this instance by a trace of the 664  $m\mu$  chromogen, retinene<sub>1</sub>, which has escaped conversion to the vitamin



A<sub>1</sub> However, the remaining eye tissues of the white perch contain both vitamins A in about equal proportions, while in the permanently fresh-water species examined they possess vitamin A<sub>2</sub> alone (see below) Consequently, in vitamin A configuration as well as in salinity relations, the white perch is distinct from all the other fishes so far mentioned It is most like the permanently fresh-water forms, as though correlated with its fresh-water origin, and shares slightly the marine type vitamin A<sub>1</sub>, as though associated with its euryhalinity

This group of fishes may be divided also on a nutritional basis, since the carp is characteristically vegetarian, the other forms carnivorous Apparently this difference in habit does not affect the retinal vitamin A configuration

Taxonomic notes on these fishes follow

Pickarel, *Esox reticulatus* Family Esocidae, the pikes, all permanently fresh-water

Calico bass, *Pomoxis sparoides* Family Centrarchidae, the sunfishes, all permanently fresh-water

Carp, *Cyprinus carpio* Family Cyprinidae, the carps and minnows This is the most extensive family of fresh-water fishes

White perch, *Morone americana* Family Serranidae, the basses

Probably the most prominent of euryhaline fishes are the anadromous salmonids, which run the gamut from the potentially to the habitually migratory, and the catadromous eel

It is now well known from the work of Schmidt (1924) that the American fresh-water eel is spawned in the Sargasso Sea (Lat 20-30° N, Long 60-80°W) The larvae migrate northward along our shores, metamorphose to elvers in the sea when about 1 year old, and at the age of 15 to 16 months ascend the rivers and streams into fresh water The eel commonly spends the subsequent 5 to 20 years in fresh water before returning to the sea to spawn

The chinook or king salmon performs the reverse migration It is spawned in fresh water, and the young parr spend from several months to 2 years in this environment before descending to the sea Adolescent or precocious individuals may thereafter reappear in fresh water in their second or third years (grilse) The mature salmon return to fresh water to spawn principally in the fourth and fifth and as late as the seventh years (Gilbert, 1912)

It is important to note that though this is the usual history, many generations of chinook and other salmon have lived permanently in

inland fresh water ponds without detriment, and it is very probable similarly that many so called fresh water eels never leave the sea.

Brook and rainbow trout, which also are salmonids, both spawn in fresh water and are indifferent in their adult habitats. Rainbow trout

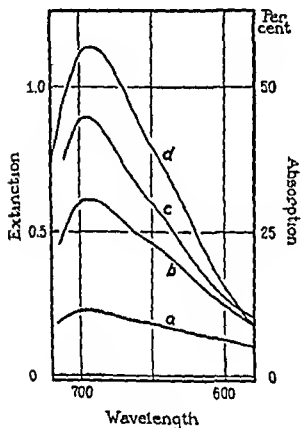


FIG 3

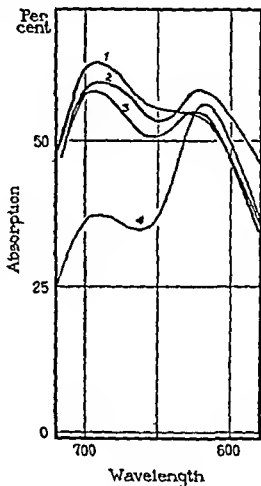


FIG 4

FIG 3 Fresh water teleost retinas. Antimony chloride reaction with total benzene extracts of (a) eight pickerel, (b) eight calico bass, (c) six white perch retinas, and (d) one carp retina. Ordinates for curves a to c are extinction, for d percentage absorption. These tissues contain only vitamin A, accompanied by traces of the unidentified 645  $m\mu$  chromogen.

FIG 4 Retinas of euryhaline fishes. Antimony chloride reaction with total benzene extracts of groups of seven retinas from (1) chinook salmon, (2) rainbow trout, (3) brook trout, and (4) eels. All of these tissues contain mixtures of vitamins A<sub>1</sub> and A<sub>2</sub>; the anadromous salmonids a predominance of the latter, and the catadromous eel a predominance of the former.

rarely leave fresh water, but sea running brook trout (sea trout) are relatively common. These three salmonids therefore typify anadromous migration in all degrees of realization.

Antimony chloride reactions with retinal extracts from these fishes



and the eel are shown in Fig 4. All four spectra show clearly the presence of mixtures of both vitamins A<sub>1</sub> and A<sub>2</sub>, the eel predominantly the former and the salmonids predominantly the latter. The accurate proportions of the vitamins A, computed as described above, are presented in Table II. They show a much greater predominance of A<sub>2</sub> over A<sub>1</sub> in the salmonids than is apparent on inspection of the figure.

The eels which have been examined were mature females, caught either in fresh water or in salt marshes along the coast at the start of their spawning migration. No significant differences in vitamin A distribution are associated with these differences in habitat. These fish had experienced both marine and fresh-water existence, and the presence in the retina of both vitamins A might be a supposed consequence of this duality of environment. In any case the vitamin A pattern, once established, appears to be permanent, since the eel retains primarily marine type vitamin A<sub>1</sub> after years of existence in fresh water.

The trout and salmonids used in these experiments were obtained from a hatchery, and had not at any time been exposed to salt water. Nevertheless their retinas contain considerable quantities of vitamin A<sub>1</sub>. These animals of known life history demonstrate a relation which probably is valid for all the euryhaline fishes examined, the presence of both vitamins A in the eye tissues precedes and is independent of duality of environment. It is a genetic property of the species, not a physiological consequence of migration.

The retinas of the euryhaline eel and salmonids possess predominantly the vitamin A ordinarily associated with the environment in which each is spawned. This is accompanied secondarily by the alternative vitamin A, as though correlated with their potentiality for life in both salt and fresh water, whether or not this potentiality has been realized.<sup>4</sup>

<sup>4</sup> The observation that the vitamin A pattern is correlated primarily with the spawning environment of these fishes is consistent with the judgment of a number of naturalists on their nature. Schmidt (1924) writes of the eel, "The *Anguilla* species, in contrast to other muraenoids, are usually termed fresh-water eels, and are reckoned among the fresh-water fishes of Europe and North America. From what we have now learned this is far from literally strict. Both from their history

### Taxonomic data for these fishes follow

Eel, *Anguilla rostrata* Family Anguillidae the true eels

The following are members of the family Salmonidae, which includes the white fishes, salmons, and trouts, all anadromous or fresh water fishes

Brook trout, *Salvelinus fontinalis*

Rainbow trout, *Salmo irideus* Among the American salmonids, this most nearly resembles the European *Salmo fario*

Chinook, King or Columbia River salmon, *Oncorhynchus tshawytscha*

### *B Combined Retina and Pigmented Epithelium*

All the foregoing experiments except that upon the sand flounder were performed with fresh isolated dark adapted retinas prepared in the laboratory. The experiments now to be reported, with one exception, concern fishes obtained several hours after death in a light adapted condition. In this state the pigmented epithelium ordinarily adheres firmly to the retina. In the tautog alone of all the fishes examined the same condition is found in the dark adapted eye, in this instance the freshly dissected retinas and pigmented epithelia were irradiated as were the isolated retinas in the preceding section. In all these cases benzine extracts of the combined tissues were prepared in the usual manner. Their antimony chloride reactions are shown in Fig 5. These results are to be interpreted with a specific caution. It is possible in this type of experiment for vitamins A from the pigmented epithelium to "swamp" those from the retina, so that small amounts of one or the other vitamin A which might have appeared in extracts of isolated retinas might not be demonstrable in extracts of the combined tissues

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and their actual manner of life, these 'fresh water eels' are true oceanic fishes, and the remarkable point in their life history is not so much the fact of their migrating out into the sea to spawn as in their leaving it in order to pass their period of growth in an environment so unusual for muraenoid fishes as fresh water." Concerning the Atlantic salmon, Goode (1903, p 445) comments "I am inclined to the view that the natural habitat of the Salmon is in the fresh waters the more so since there are so many instances—such as that of the Stormontfield Ponds in England—where it has been confined for years in lakes without apparent detriment." That the chinook salmon has been kept for years in fresh water ponds in France is another strong evidence of the correctness of this view (Jordan and Evermann, 1902)

The marine haddock, whiting, and herring eye tissues all contain vitamin A<sub>1</sub> alone. The herring forms with the alewife a pair similar to the sea bass and white perch. The former fish belong to one family and resemble each other closely anatomically. The herring is permanently marine, but the alewife enters fresh water to spawn. Immediately afterward it returns to the sea, and since the young fry migrate seaward within several months after hatching, ordinarily this fish spends only a few months of its entire existence in fresh water. Still its eye contains predominantly, perhaps exclusively, the fresh-water fish type vitamin A<sub>2</sub>. This is a striking added demonstration that the vitamin A configuration of the eye tissues is principally and permanently associated with the spawning environment.<sup>5</sup>

The data so far presented reveal so precise a correlation between vitamin A distribution and salinity relations as to sharply accentuate the case of the tautog. The tautog alone of all the fishes examined possesses a vitamin A pattern the reverse of that found generally.

The combined retinas and pigmented epithelia of several dark adapted tautogs were extracted *in darkness* three times with benzene in order to remove stored vitamins A and permit the retinal changes following irradiation to be estimated. The first two extracts contained a moderate quantity of vitamin A<sub>2</sub>, the third only a faint trace of this vitamin. The tissue was exposed to bright light and left in moderate light at room temperature for about 1½ hours. A fourth benzene extract now yielded curve *e* of Fig 5. It shows the formation, during bleaching, of a large quantity of new vitamin A<sub>2</sub> accompanied by a small amount of A<sub>1</sub>. Similar results have been obtained previously only from anadromous fishes. Yet the tautog is permanently marine, as are also all members of its family, the Labridae. The possible significance of this situation is discussed below. Taxonomic data on the fishes just reviewed follow.

Haddock, *Melanogrammus aeglefinus* Family Gadidae, the cods

Whiting or silver hake, *Merluccius bilinearis* Family Merluccidae, the silver hakes

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<sup>5</sup> The alewife, like the salmonids and white perch, may spend its entire existence in fresh water without detriment. Large numbers are found, apparently land-locked, in Lake Ontario and certain small lakes in New York State (Jordan and Evermann, 1902).

The sea berring *Clupea harengus*, and the alewife, *Pomolobus pseudoharengus*, are members of the same family, the Clupeidae

Tautog, *Tautoga onitis* Family Labridae, the cunners or wrassefishes

### C Choroid and Pigmented Epithelium

The pigmented layers of all the marine fishes examined contain vitamin A<sub>1</sub> alone. Data from the herring and sand flounder are shown in Fig 6 (c, d), and from the smooth and spiny dogfishes in Fig 2 (b, d). The latter display small amounts of the 664 mμ chromogen, retinene<sub>1</sub>. This substance has not previously been found in the pigmented layers, and its significance in this situation in the dogfishes is problematical.

Antimony chloride tests with pigmented layer extracts from the fresh water carp and calico bass are shown in Fig 6 (a, b). They reveal no trace of vitamin A<sub>1</sub>, but instead high concentrations of A<sub>2</sub>, accompanied by the unknown 645 mμ chromogen. The latter is more prominent in these extracts than in the retinal oils.

Similar tests with extracts from euryhaline fishes are shown in Fig 7. Curves a, b, and c from the salmonids demonstrate mixtures of vitamins A<sub>1</sub> and A<sub>2</sub> with a clear predominance of the latter. Curve d, from the eel, also displays the bands of both vitamins A, apparently in reverse proportion to the salmonids. The computed proportions of vitamins A in all these extracts are presented in Table II, they show the "color equivalents" of A<sub>1</sub> and A<sub>2</sub> to be about equal in the eel tissues.

White perch pigmented layers yield a peculiar result (Fig 7e). The test spectrum is dominated by the 645 mμ band which heretofore has appeared only as a faint satellite of the 696 mμ absorption. Low bands due to vitamins A<sub>1</sub> and A<sub>2</sub> may also be distinguished, but their proportions cannot be estimated in this preparation. A similar result has been obtained by Edisbury *et al* (1938) with a liver oil from the true perch (*Perca fluviatilis*).

The pigmented layers, therefore, present about as precise correlation of vitamin A pattern with salinity relations as do the retinas. This observation lessens the force of our distinction between the retina proper, practically all of whose vitamins A are directly involved in the visual processes, and the extra retinal tissues in which vitamins A seem merely to be stored. Apparently the accumulation of vitamins

A may be a highly selective process even in tissues which contain much larger amounts than they appear to utilize. The vitamin A

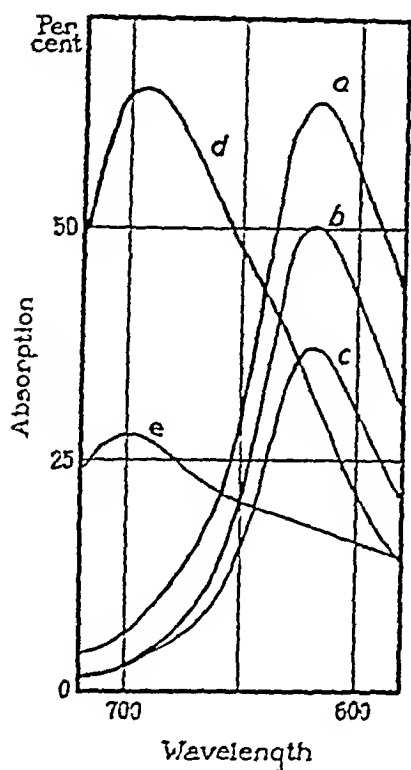


FIG 5

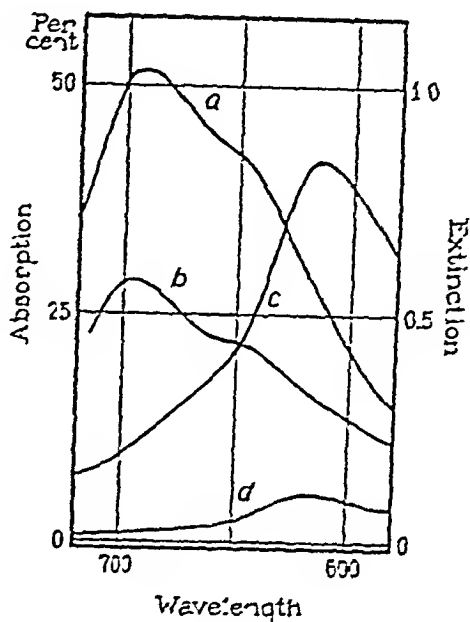


FIG 6

FIG 5 Combined retinas and pigmented epithelia. Antimony chloride reactions with benzine extracts from the marine (a) herring, (b) whiting, (c) haddock, and (e) tautog, and (d) from the anadromous alewife. The predominance of vitamin A<sub>2</sub> in the tautog tissues is the reverse of the results obtained with all the other marine fishes examined.

FIG 6 Pigmented epithelia and choroids of stenohaline fishes. Antimony chloride reactions with chloroform extracts of the combined tissues from (a) four carp, (b) eight calico bass, (c) one sand flounder, and (d) of the choroids from seven herring. Ordinates of curves a, c, and d are per cent absorbed, for b are extinction. Tissues from the marine fishes contain vitamin A<sub>1</sub> alone, those from the fresh-water fishes only vitamin A<sub>2</sub>.

configuration not only of the retina but of all the tissues of the ocular fundus is a genetic characteristic, primarily independent of the immediate environment and life history of the fishes examined.

*D Liver*

The vitamin A pattern and salinity relations are not correlated as precisely in the liver as in the eye tissues. Most liver oils contain mixtures of both vitamins A, occasionally in proportions the reverse

TABLE II

Proportions of vitamins A<sub>1</sub> and A<sub>2</sub> in fish eye tissues and livers. These are stated in terms of "color equivalents" the extinction at 618 mμ due to vitamin A<sub>1</sub> compared with the extinction at 696 mμ due to vitamin A<sub>2</sub>. Each pair of values is summed to 100, so that each figure has the force of a percentage

Type	Fish	Retinas		Other eye tissues		Livers	
		A <sub>1</sub>	A <sub>2</sub>	A <sub>1</sub>	A <sub>2</sub>	A <sub>1</sub>	A <sub>2</sub>
Marine	Sea bass	100	0	—	—	—	—
	Scup	100	0	—	—	—	—
	Sea robin	100	0	—	—	—	—
	Sand flounder	100	0	100	0	94	6
	Smooth dogfish	100	0	100	0	—	—
	Spiny dogfish	100	0	100	0	100	0
	Herring	100*	0	100	0	80	20
	Haddock	100*	0	—	—	—	—
	Whiting	100*	0	—	—	—	—
	Tautog	18*	82	—	—	—	—
	Halibut	—	—	—	—	92	8
Fresh water	Cod	—	—	—	—	100	0
	Pickereel	0	100	—	—	0	100
	Calico bass	0	100	0	100	0	100
	Carp	0	100	0	100	65-75	25-35
Anadromous	White perch	0	100	?	?	—	—
	Alewife	0*	100	—	—	19	81
	Chinook salmon	29	71	20	80	75-80	20-25
	Rainbow trout	38	62	30	70	41	59
	Brook trout	35	65	30	70	90	10
Catadromous	Eel	62	38	50	50	99	1

\* Pigmented epithelium included

of those in the eye. The relative amounts of vitamins A<sub>1</sub> and A<sub>2</sub> in fish livers, computed as described above, are presented in the last two columns of Table II. Similar data on the liver oils of a large number of European fishes appear in the papers of Edisbury *et al* (1938), Gillam *et al* (1938), and Lederer and Rathmann (1938).

Let us now consider the potassium effect, *i.e.*, the change in P D produced by substituting KCl for NaCl. This was measured as shown in Fig 4. Before the application of HG this is  $94.4 \pm 2.1$  mv (22 observations). As explained in previous papers<sup>10</sup> this may be calculated by means of Henderson's equation if we know the values of  $u_{Na}$  and  $u_K$ . Before the application of HG we have  $u_K = 8.76$  and  $u_{Na} = 2.33$  (p 420) and we find that we must put  $S_K - S_{Na} = 38$  to get the observed<sup>30</sup> P D of  $94.4$  mv. After the application of HG we have  $u_K = 8.76$  and  $u_{Na} = 7.30$  (p 420) and we must then put  $S_K - S_{Na} = 2.35$  to get the observed value<sup>31</sup> of  $20.9$ . Since  $S_K$  is not changed by HG, as is shown by the behavior of the curve in Fig 5, we may suppose that HG has multiplied the value of  $S_{Na}$  by 16.2 which would reduce the ratio from 38 to 2.35. (We have already seen that HG changes  $u_{Na}$  from 2.33 to 7.30.)

We may conclude that HG increases  $u_{Na}$  and  $S_{Na}$  with little or no effect on  $u_K$  or  $S_K$ .

This recalls the situation in *Halicystis* where HG appears to increase  $u_{Na}$  without affecting  $u_K$ . In *Valonia*<sup>1</sup>  $u_{Na}$  is increased and  $u_K$  is decreased.<sup>32</sup>

The changes produced by HG in the concentration effect and in the potassium effect are to some extent predictable from the electrical behavior of HG. Preliminary experiments in this laboratory, in collaboration with J. W. Murray, show<sup>33</sup> that when the *Nitella* cell is

<sup>30</sup> The value for the change from 0.01 M NaCl to 0.01 M KCl and *vice versa* is  $94.4 \pm 2.1$  (22 observations).

<sup>31</sup> The value for the change from 0.01 M NaCl to 0.01 M KCl and *vice versa* is  $20.9 \pm 0.7$  mv (32 observations) this is after the action current has been produced as in Fig 4, in the presence of NaCl. When the action current has been produced as in Fig 6, in the presence of KCl, the corresponding value is  $24.7 \pm 1.4$  mv (6 observations).

<sup>32</sup> Cf footnote 1. An exposure of about 5 minutes is necessary to produce this result. But it does not occur in *Nitella* or in *Halicystis* even with an exposure of 10 minutes to 0.02 M HG.

<sup>33</sup> In measuring the concentration effect the HG was first shaken with the more dilute solution and left in contact with it in a U-tube, the more concentrated solution was then placed in contact with the other surface of the HG. The two aqueous solutions were then connected through calomel electrodes to a Compton electrometer. The sign in all cases is that observed in the external circuit.

In measuring the potassium effect the HG was shaken with NaCl and KCl was then placed on the opposite side.

replaced by a liquid layer of HG the concentration effect of NaCl is somewhat smaller than that of KCl, which agrees with *Nitella*. The dilute solution is positive in both cases, as in *Nitella*.

For the potassium effect (0.1 M NaCl vs 0.1 M KCl) the value is about 14 mv. The KCl is negative to NaCl, as in *Nitella*.

Since the potassium effect is less in HG than in *Nitella* we might expect the addition of HG to the non aqueous protoplasmic surface layer, X, to lessen the value of the potassium effect, as is indeed the case. And since the concentration effects of KCl and NaCl are nearer together in HG than in *Nitella* we might expect the addition of HG to bring these effects nearer together in *Nitella*, as is the case.

The fact that  $\mu_{Na}$  is increased in *Nitella* and  $\mu_K$  left unaffected is not explained by these considerations. Further investigation is needed.

It is evident that the action of HG cannot be accounted for on the ground that the protoplasmic surface is a pore system since a change in the pores could not increase  $\mu_{Na}$  and leave  $\mu_K$  unaffected as in *Nitella* and *Halicystis* or increase  $\mu_{Na}$  and simultaneously decrease  $\mu_K$  as in *Valonia*.

#### SUMMARY

In *Nitella*, as in *Halicystis*, guaiacol increases the mobility of  $Na^+$  in the outer protoplasmic surface but leaves the mobility of  $K^+$  unaffected. This differs from the situation in *Valonia* where the mobility of  $Na^+$  is increased and that of  $K^+$  is decreased.

The partition coefficient of  $Na^+$  in the outer protoplasmic surface is increased and that of  $K^+$  left unchanged.

Recovery after the action current is delayed in the presence of guaiacol and the action curves are "square topped."





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# FORMATION OF TRYPSIN FROM CRYSTALLINE TRYPSINOGEN BY MEANS OF ENTEROKINASE

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The mechanism of formation of trypsin from trypsinogen by means of enterokinase has been a matter of almost continuous controversy since the discovery of enterokinase by Schepowalnikow in Pawlow's laboratory in 1899. Pawlow and Schepowalnikow considered enterokinase to be a typical enzyme. Studies of the kinetics of activation have shown, however, that while the rate of activation of a definite concentration of trypsinogen was proportional to the concentration of enterokinase used, the ultimate amount of trypsin formed was not independent of the concentration of enterokinase, as would be expected were enterokinase a true enzyme. This led to the suggestion that the formation of trypsin from trypsinogen by enterokinase is essentially a stoichiometric combination between trypsinogen and enterokinase to form an active enzyme, "trypsin kinase" (1).

The isolation (8) of crystalline trypsinogen from fresh beef pancreas, its autocatalytic transformation at pH 7.0-9.0 into active trypsin without the aid of any outside activator, and the isolation of the active trypsin in pure crystalline form offer proof against the assumption that trypsin is a stoichiometric compound of kinase and trypsinogen. It was also found that pure crystalline trypsinogen can be changed into active trypsin at pH 3.0-4.0 by means of a kinase obtained from a mold of the genus *Penicillium* (2). The trypsin formed was crystallized and found to be identical in crystalline form, solubility, and specific activity with the crystalline trypsin obtained by spontaneous autocatalytic activation of trypsinogen at pH 8.0. The action of mold kinase was that of a typical enzyme, the process of activation following the course of a catalytic unimolecular reaction and the ultimate amount of trypsin formed being independent of the concentration of mold kinase used.

This paper deals with the kinetics of the formation of trypsin from crystalline trypsinogen by means of purified enterokinase obtained from swine duodenum contents. Enterokinase acts best in the range of pH 6.0–9.0 where autocatalytic formation of trypsin from trypsinogen occurs readily. The percentage rate of this reaction, however, is proportional to the concentration of trypsinogen. Hence, by using very dilute trypsinogen solutions the rate of the autocatalytic activation may be made negligible compared with that of the activation brought about by a significant amount of enterokinase (3). The autolysis of the trypsin formed, which generally occurs in the range of pH 7.0–9.0 (4), is also minimized by using dilute trypsinogen and by employing temperatures not higher than 5°C.

A further complication exists at pH 7.0–9.0 since under these conditions trypsinogen in the presence of trypsin is partly changed to an inert protein (5) which can no longer be transformed into trypsin either by enterokinase or mold kinase. This complication is minimized in solutions more acid than pH 6.0 where the rate of transformation of trypsinogen into inert protein is greatly reduced. When activation by enterokinase is allowed to proceed at pH below 6.0 enterokinase acts almost like a typical enzyme. The reaction follows approximately the course of a theoretical unimolecular reaction with a velocity constant proportional to the concentration of enterokinase used and the ultimate amount of trypsin formed is practically independent of the concentration of kinase.

If, on the other hand, the activation is allowed to proceed at pH above 6.0 a great portion of the trypsinogen is transformed into inert protein, the more so the lower the concentration of enterokinase used. As a result, the ultimate amount of trypsin formed is less as the concentration of enterokinase used is decreased in agreement with the findings of earlier workers (6).

The kinetics of the formation of the trypsin from crystalline trypsinogen by means of enterokinase under conditions where part of the trypsinogen is changed into inert protein can be derived mathematically as follows

Let  $G_0$  = initial concentration of trypsinogen

$E$  = concentration of enterokinase

$A$  = concentration of trypsin at any time  $t$

$A_0$  = final concentration of trypsin

$I$  = concentration of inert protein formed from trypsinogen in any time  $t$

$I_0$  = final concentration of inert protein

$G_0 - A - I$  = concentration of trypsinogen at any time  $t$

Assuming (1) that the rate of formation of trypsin is proportional to the concentration of enterokinase and to the concentration of trypsinogen and, (2) that the rate of formation of inert protein is proportional to the concentration of trypsin and to the concentration of trypsinogen, we have the following equations

$$\frac{dA}{dt} = K E(G - A - I) \quad (1)$$

$$\frac{dI}{dt} = K_1 A(G - A - I) \quad (2)$$

Hence

$$\frac{dI}{dA} = \frac{K_1 A}{K E} \quad (3)$$

and

$$I = bA^2 \quad (4)$$

where  $b = \frac{K_1}{2KE}$  and  $K_1$  and  $K$  are the velocity constants of the reactions

Substituting  $bA^2$  for  $I$  in Equation 1, we get

$$\frac{dA}{dt} = K E(G - A - bA^2) \quad (5)$$

At the end of the reaction when  $\frac{dA}{dt} = 0$  we have

$$G = A + bA^2, \quad (6)$$

or

$$\frac{G - A}{A^2} = b = \frac{K_1}{2KE} \quad (7)$$

Substituting  $A_e + bA_e^2$  for  $G_o$  in Equation 5 we get

$$\frac{dA}{dt} = K_e E [(A_e - A)(1 + bA_e + bA)] \quad (8)$$

which on integration gives

$$\ln \frac{A_e}{A_e - A} + \ln \left( 1 + \frac{bA}{1 + bA_e} \right) = mt \quad (9)$$

where

$$m = K_e E + K_2 A_e \quad (10)$$

The exponential form of Equation 9 is

$$A = \frac{A_e(e^{mt} - 1)}{e^{mt} + \frac{bA_e}{1 + bA_e}} \quad (11)$$

where  $m$  is the slope of the straight line obtained when the values for

$$\ln \frac{A_e}{A_e - A} + \ln \left( 1 + \frac{bA}{1 + bA_e} \right)$$

are plotted against  $t$ , in accordance with Equation 9

It follows from Equations 7 and 10 that

$$K_e E = \frac{mA_e}{2G_o - A_e} \quad (12)$$

and

$$K_2 = \frac{m - K_e E}{A_e} \quad (13)$$

At pH more acid than 6.0  $K_2$  becomes negligible and Equations 9 and 11 are then reduced to the approximate forms

$$\ln \frac{G_o}{G_o - A} = K_e E t \quad (9a)$$

and

$$A = G_o(1 - e^{-K_e E t}) \quad (9b)$$

which are the equations of a simple catalytic unimolecular reaction. If an appreciable amount of trypsin,  $A_o$ , is present as an impurity in the sample of trypsinogen used then Equation 9 becomes

$$\ln \frac{A_e - A_o}{A_e - A} + \ln \frac{1 + bA_e + bA}{1 + bA_e + bA_o} = mt$$

where

$$b = \frac{G + A - A^2}{A^2 - A^1}$$

also Equation 4 becomes

$$I = b(A^2 - A^1)$$

The derived equations bring out the following relationship between the trypsin and inert protein formed from trypsinogen in the presence of enterokinase at pH above 6.0: the concentration of inert protein formed at any time during the reaction is proportional to the square of the concentration of trypsin formed and inversely proportional to the concentration of enterokinase used (Equation 4).

It follows then that the higher the concentration of enterokinase used the greater is the percentage of trypsinogen changed into active trypsin.

This relationship, as well as the equation for the kinetics of the enterokinase activation, has been found to check closely with the experimental results.

The transformation of trypsinogen into trypsin in the presence of enterokinase appears thus to be a typical enzyme reaction catalyzed by the enzyme enterokinase. The anomalous results found under certain conditions are due to a secondary reaction by which trypsin changes trypsinogen to an inert protein.

The kinetics of the reaction outlined above applies only to dilute solutions of purified trypsinogen. The activation of concentrated solutions of trypsinogen is complicated by rapid autocatalytic formation of trypsin by the trypsin itself. The activation of crude pancreatic extracts is much more complicated since, as previously noted (7), these extracts contain chymo trypsinogen in addition to trypsinogen and also a substance which inhibits trypsin (8). In outline the activation of crude trypsinogen by enterokinase proceeds as follows: Addition of kinase transforms the trypsinogen to trypsin which catalyzes the conversion of trypsinogen to form more trypsin and which also catalyzes the conversion of chymo trypsinogen to chymo trypsin. If the method of activity determinations used determines both trypsin and chymo trypsin, as is usually the case, the curves obtained when the activity of the solution is plotted against time are S shaped but



asymmetrical and resemble those obtained by Vernon (9) These curves generally show a long initial lag period which is partly caused by the interference of the trypsin inhibitor with the catalytic action of the trypsin formed

*Experimental Studies of the Kinetics of the Formation of Trypsin from Crystalline Trypsinogen by Means of Enterokinase General Procedure*

Reaction mixtures were made up of solutions of crystalline trypsinogen and of enterokinase in dilute buffers and allowed to stand at 5°C The solutions were kept sterile by the addition of 0.1 ml 1 per cent merthiolate in 1.4 per cent borax solution to 100 ml of reaction mixture Samples of 1 ml were acidified with hydrochloric acid to about pH 2.0 in order to stop the reactions The concentration of trypsin in the samples was then determined by the hemoglobin method of Anson (10) Samples were also taken in some cases for the determination of the concentration of inert protein formed during the reaction by the method described elsewhere (11) and which consists essentially in adding to the samples a large excess of enterokinase and thus bringing about rapid and complete activation of all the available trypsinogen

*Kinetics of Formation of Trypsin by Means of Enterokinase at pH 5.6 and 7.6*—The striking difference in the behavior of enterokinase when allowed to act on crystalline trypsinogen at pH 5.6, as compared with that of pH 7.6, is shown in Fig. 1 At pH 5.6 enterokinase acts almost like a typical enzyme so that the ultimate concentration of trypsin formed in a solution of trypsinogen of a definite concentration varies only slightly with the concentration of enterokinase used, while at pH 7.6 the ultimate concentration of trypsin formed varies markedly with the concentration of enterokinase

Fig. 2 shows the experimental data for the action of enterokinase on crystalline trypsinogen at pH 5.6 plotted logarithmically (Equation 9a)

$$\ln \frac{G_0}{G_0 - A} \text{ vs. } t$$

In the calculations the value of  $G_0$  was taken as  $1.5 \times 10^{-3}$  [T U]<sup>HB</sup> per ml which is the value obtained in the presence of a large excess of enterokinase In the presence of the concentrations of kinase used in this experiment (Fig. 1, pH 5.6) the activity reaches a maximum value of only  $1.3\text{--}1.35 \times 10^{-3}$  [T U]<sup>HB</sup> per ml, the difference being

due to the formation of a small amount (10-20 per cent) of inert protein. In the first part of the reaction this formation of inert

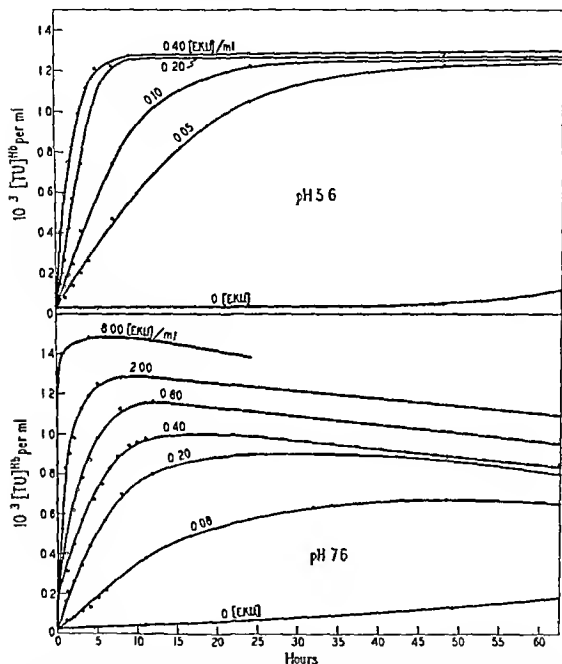


FIG 1 Formation of trypsin from crystalline trypsinogen by enterokinase at 5°C. Activation mixtures 5 ml 0.065 per cent solution of trypsinogen in 0.005 M hydrochloric acid + 10 ml 0.1 M phosphate buffer + 1.0 ml enterokinase solution in water + distilled water to 50 ml

protein is too small to affect the results but in the latter part of the reaction when  $A$  approaches the value of  $G$  the reaction will proceed more slowly than calculated on the simple assumption that no inert

protein is formed. Thus in Fig 2, where the results have been plotted by the simple monomolecular equation the theoretical and experimental points agree up to 50–60 per cent activation and the slopes of the lines are proportional to the concentrations of enterokinase used. In the last 30–40 per cent of the reaction the formation of inert protein becomes significant and the experimental points lie below those predicted by the simple equation in which the formation of inert protein was neglected.

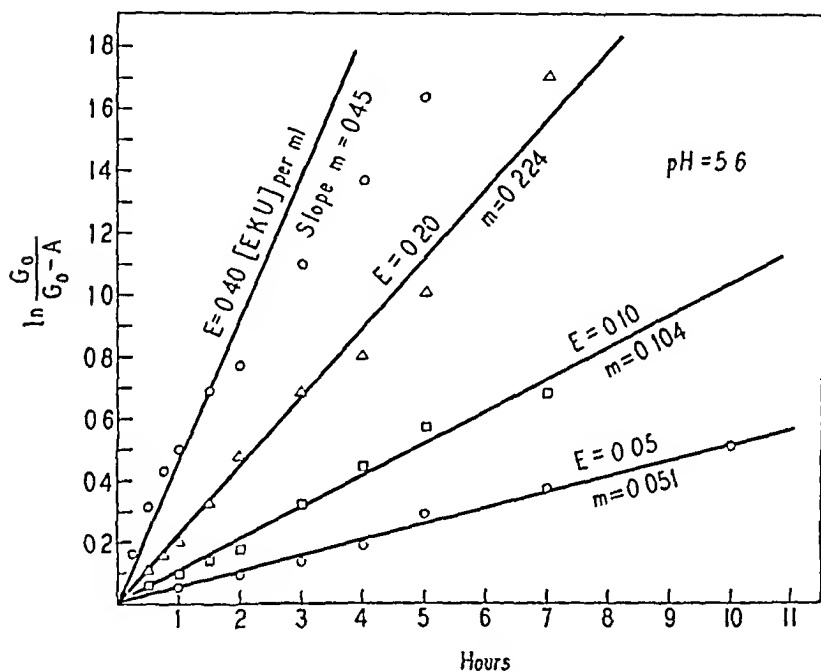


FIG 2 Logarithmic curves of Fig 1 pH 5.6 plotted in accordance with the equation of a simple unimolecular reaction

On the other hand, curve I in Fig 3 shows that the experimental data for the action of enterokinase at pH 7.6 do not fall in straight lines when plotted logarithmically in accordance with the theoretical equation of a simple unimolecular reaction even in the first part of the reaction. The experimental points, however, do fall in a straight line (curve II, Fig 3) when plotted according to the more complete Equation 9 which takes care of the complication due to the formation of inert protein. Curve III of Fig 3 shows the close agreement between

the observed and theoretical values of  $A$ . The last were computed by means of Equation 11 with  $m = 0.21$  as given by the slope of curve II. The logarithmic curves (Equation 9) for the whole series of pH 7.6 are shown in Fig. 4. In practically all cases the experi-

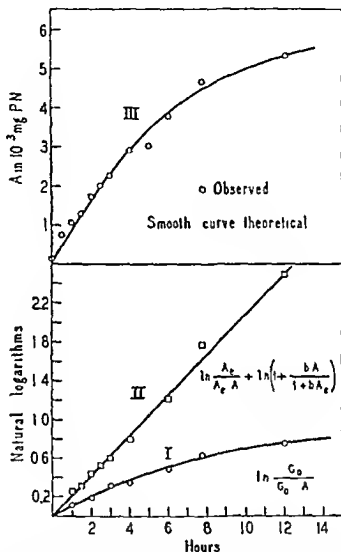


FIG. 3. Comparison between theoretical and observed data on the formation of trypsin from crystalline trypsinogen by enterokinase at pH 7.6 and 5°C. Concentration of trypsinogen 0.01 mg protein nitrogen per ml. Concentration of enterokinase 0.20 [E. K. U.] per ml.

mental points lie on straight lines. The slopes of the various lines, as well as the calculated values of  $K_1$ ,  $K_2$ , and  $A$ , are given in Table I. The concentrations of  $G$  and  $A_e$  are expressed in mg protein nitrogen per ml, 1 mg protein nitrogen being equivalent to 0.15 [T. U.]<sup>11b</sup>.

**Formation of Inert Protein.**—Fig. 5 shows the time curves for the

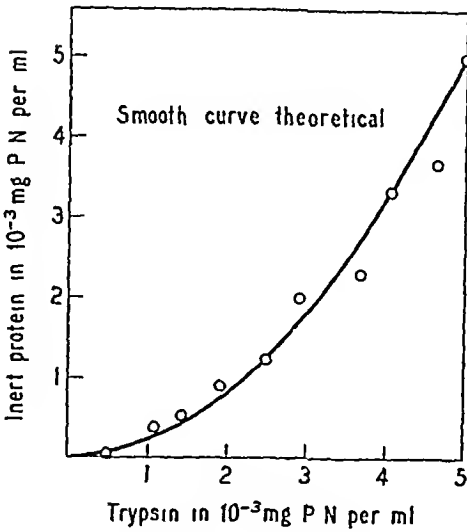


FIG 6 Relation between trypsin and inert protein formed from crystalline trypsinogen at pH 7.6 and 5°C in the presence of enterokinase. The smooth curve is the theoretical locus of the equation  $I = bA^2$  where  $b = \frac{G_0 - A_e}{A^2} = 200$

TABLE II  
*Effect of pH*

Activation mixture 1 ml 1 M  $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$  mixtures, plus 1.0 ml enterokinase, 5 [E K U] per ml water, plus 5.0 ml crystalline trypsinogen, 0.1 mg protein nitrogen per ml M/200 hydrochloric acid, plus water to 50 ml. Samples 1.0 ml plus 1.0 ml 0.04 M hydrochloric acid for activity measurements.

Trypsinogen concentration  $G_0 = 0.01$  mg protein nitrogen per ml

Enterokinase concentration  $E = 0.1$  [E K U] per ml

pH	5.26	5.78	6.12	6.38	6.55	6.72	6.94	7.18	7.45	7.75
$A_e$ in $10^{-3}$ mg trypsin protein nitrogen per ml	8.1	7.3	6.1	5.6	4.6	4.3	3.8	3.4	3.2	2.9
$K_e E + K_2 A_e$ per hr (from logarithmic plot Equation 9)	0.053	0.13	0.20	0.24	0.27	0.25	0.30	0.35	0.36	0.40
$K_e$ per [E K U] per hr (Equation 12)	0.36	0.75	0.87	0.93	0.79	0.68	0.71	0.72	0.68	0.67
$K_2$ per mg trypsin protein nitrogen per hr (Equation 13)	2	8	19	26	41	43	60	82	93	113

in the presence of enterokinase  $I$  is proportional to the *square* of  $A$ . Both relations, however, are derived mathematically on the basis of the same assumption that the formation of inert protein is catalyzed by the trypsin formed.

*Effect of pH on the Velocity Constants of Both Reactions*

The striking difference in the kinetics of formation of trypsin by means of enterokinase at pH 5.8 and at pH 7.6 was shown in Fig 1. A summary of a series of experiments on the effect of pH in the region of 5.26–7.75 on the kinetics of formation of trypsin from trypsinogen in the presence of enterokinase at 7°C is given in Table II and also in Fig 7.

The results show that there is a gradual decrease in the amount of trypsin formed with the increase of pH. At pH more acid than 5.0

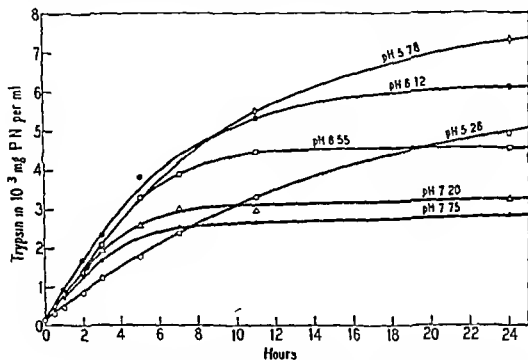


FIG 7 Formation of trypsin at 7°C by means of enterokinase at various pH.  $G = 0.01$  mg trypsinogen protein nitrogen per ml. Enterokinase =  $0.1$  [E.K.U.] per ml.

there is, however, a sudden drop in the amount of trypsin formed due to destruction of enterokinase. The optimum range of pH for the rate of formation of trypsin by means of enterokinase is at about 6.2 as shown on curve K, Fig 8. The values of  $K_2$  for the various pH, as calculated from the slopes of the logarithmically plotted curves, are of the same magnitude as those obtained under the same approximate conditions of temperature, salt, and trypsinogen concentration in the absence of enterokinase during the autocatalytic formation of trypsin (13).

*Effect of Concentration of Trypsinogen*

With increase in concentration of trypsinogen in solution, the concentration of enterokinase being kept constant, the rate of the catalytic formation of inert protein is greatly increased as compared with the rate of the catalytic formation of trypsin by enterokinase, since the relative rate of formation of the two products is proportional to the

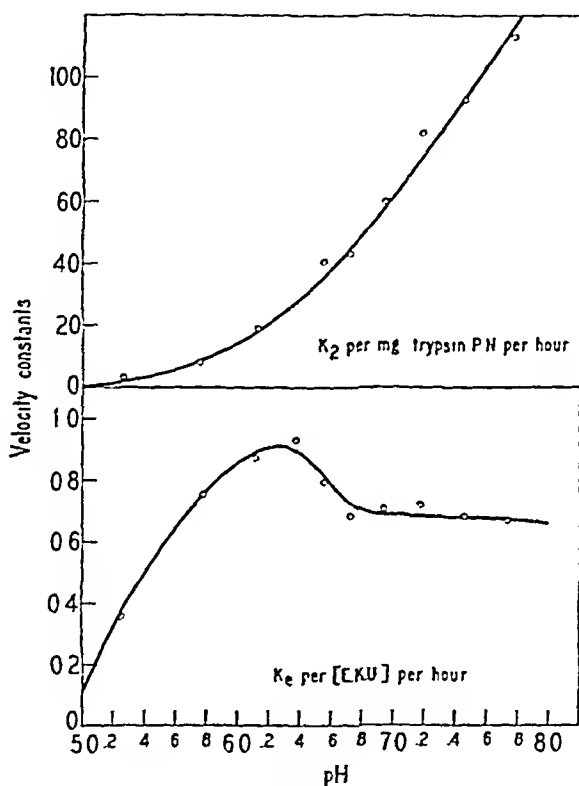


FIG 8 Effect of pH on the velocity constants

concentration of trypsin formed (Equation 3) The complicating effect of the formation of inert protein on the kinetics of the enterokinase action as the concentration of trypsinogen is increased becomes evident even at pH 5.8 This is shown in Fig 9 where the percentage of trypsinogen changed into trypsin was plotted against  $t$  The higher the concentration of trypsinogen used the lower was the percentage rate as well as the final per cent of trypsinogen changed into trypsin

The results of the experiment are summarized in Table III where

the observed values of inert protein formed are given. In every case the sum of values of  $A_0$  and  $I_0$  is equal to the corresponding value of  $G_0$ .

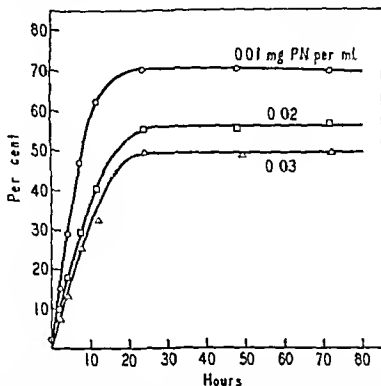


FIG 9 Effect of concentration of trypsinogen on the formation of trypsin from crystalline trypsinogen by means of enterokinase at pH 5.8 and 6°C. Concentration of enterokinase 0.1 [E K U] ml

TABLE III

*Effect of Concentration of Trypsinogen at pH 5.8 and 6°C*

Concentration of enterokinase 0.08 [E K U] per ml 0.02 M phosphate buffer pH 5.8 of activation mixture

Concentration of trypsinogen = $G$ in mg protein nitrogen per ml activation mixture	0.01	0.02	0.03
Final concentration of trypsin formed = $A$ in trypsin mg protein nitrogen per ml	0.007	0.011	0.015
Final concentration of inert protein formed = $I$ (observed) in mg protein nitrogen per ml	0.003	0.008	0.015
$\frac{K_2}{K} = \frac{2E(G - A)}{A^2}$	10	12	11

The table also shows that the relation between  $G$  and  $A$  in each case checks with the theoretical Equation 7 giving a value of  $\frac{K_2}{K}$



independent of the original concentration of trypsinogen used. The values of  $K_e$  and  $K_2$  as calculated from the slopes of the logarithmic curves appear, however, to decrease with increase in concentration of trypsinogen. A similar effect of increase in concentration of substrate on the magnitude of the velocity constant has been frequently observed in the case of enzymatic reactions (14).

Addition of fresh trypsinogen to the activation mixtures at the end of the reaction always brought about formation of more trypsin, thus proving that the incomplete activation of the original trypsinogen was not due to any insufficiency or possible inactivation of the enterokinase. On the other hand, the addition of excess enterokinase or mold kinase to the activation mixture at the end of the reaction has never brought about an increase in the concentration of trypsin although no significant loss of protein has been noticed. It is evident that the incompleteness of the enterokinase reaction is due to the partial transformation of the trypsinogen into inert protein which cannot be changed into trypsin by any known activator.

### Methods

1 *Preparation of Crystalline Trypsinogen*—The trypsinogen was prepared by the method of Kunitz and Northrop (8). The crystals were purified and made inhibitor free by means of trichloroacetic acid as described on page 993 of the same reference, except for an extra step in the process which was omitted through a typographical error. The corrected procedure for purification by means of trichloroacetic acid is as follows: 10 gm. filter cake of trypsinogen crystals is dissolved in 200 ml.  $N/400$  hydrochloric acid and 200 ml. 5 per cent trichloroacetic acid added. The solution is left at  $20^\circ C$  for 1 hour and then filtered with suction and washed several times with small amounts of 2.5 per cent trichloroacetic acid and finally with water. The semi-dry precipitate is dissolved in 25 times its weight of  $N/50$  hydrochloric acid, allowed to stand about 30 minutes. Ammonium sulfate is added to 0.4 saturation. The precipitate is filtered off and rejected. The filtrate is brought to 0.7 saturation with solid ammonium sulfate and filtered with suction. The filter cake is dissolved in 3–5 times its weight of  $N/200$  hydrochloric acid and dialyzed for 24 hours at  $5-6^\circ C$  against running  $N/200$  hydrochloric acid.

2 *Preparation of Enterokinase*—A stock of enterokinase in water and containing about 1,000 kinase units per ml. was prepared from intestinal contents by the method described elsewhere (15).

3 *Estimation of Enterokinase*—The quantity of enterokinase in any solution is expressed in terms of the velocity with which it transforms crystalline trypsinogen.

gen into trypsin under standard conditions. One enterokinase unit, 1 [E K U] is the amount of kinase that brings about the activation of 0.065 mg crystalline trypsinogen (0.01 mg protein nitrogen) in 0.02 M Sørensen's phosphate buffer pH 5.8 at the rate of 100 per cent per hour at 5°C. Under these conditions the activation by enterokinase follows approximately the course of a simple unimolecular reaction so that the plotted values of  $\ln \frac{G}{G-A}$  vs  $t$  fall in straight lines (Fig. 2) the slopes of which are proportional to the concentration of enterokinase used. The concentration of enterokinase in each case can be taken as equal to the slope of the lines by assigning the value of unity to the proportionality constant.

The standard method of estimating kinase involves the determination of concentration of trypsin in a series of samples taken at various intervals of time from the activation mixture in order to obtain several points for the logarithmic curve. For practical purposes the following simplified procedure was adopted. *Activation mixture*: 3 ml 0.02 M phosphate buffer pH 7.6 plus 1.0 ml enterokinase in 0.02 M phosphate pH 7.6 plus 1 ml standard crystalline trypsinogen solution (0.1 mg protein nitrogen per ml) in N/200 hydrochloric acid. The activation mixture is placed for 30 minutes in a water bath at 25°C. 1.0 ml of the mixture is then added to 5.0 ml Anson's urea hemoglobin solution; its trypsin content  $[T U]^{Hb}$  is determined as described by Anson (10). The concentration of enterokinase in [E K U] per ml activation mixture corresponding to the  $[T U]^{Hb}$  measured is then read off a standard curve. The standard curve is obtained by plotting the data of  $[T U]^{Hb}$  vs [E K U] for a series of activation mixtures containing various dilutions of a stock of enterokinase of known [E K U] content, as determined by the standard method. 1 [E K U] is equivalent to about 100 mg of acetone dried pigs duodenal mucosa.

4 *Estimation of Trypsin*—Method of Anson (10)

5 *Estimation of Inert Protein*—Described in preceding paper (11)

The writer was assisted by Margaret R. McDonald

### SUMMARY

Crystalline trypsinogen is most readily and completely transformed into trypsin by means of enterokinase in the range of pH 5.2–6.0 at 5°C and at a concentration of trypsinogen of not more than 0.1 mg per ml. The action of enterokinase under these conditions is that of a typical enzyme. The process follows closely the course of a catalytic unimolecular reaction, the rate of formation of trypsin being proportional to the concentration of enterokinase added and the ultimate amount of trypsin formed being independent of the concentration of enterokinase.

The catalytic action of enterokinase on crystalline trypsinogen in

dilute solution at pH more alkaline than 6.0 and in concentrated solution at pH even slightly below 6.0 is complicated by the partial transformation of the trypsinogen into inert protein which can no longer be changed into trypsin even by a large excess of enterokinase. This secondary reaction is catalyzed by the trypsin formed and the rate of the reaction is proportional to the concentration of trypsin as well as to the concentration of trypsinogen in solution. Hence under these conditions only a small part of the trypsinogen is changed by enterokinase into trypsin while a considerable part of the trypsinogen is transformed into inert protein, the more so the lower the concentration of enterokinase used.

The kinetics of the formation of trypsin by means of enterokinase when accompanied by the formation of inert protein can be explained quantitatively on the theoretical assumption that both reactions are of the simple catalytic unimolecular type, the catalyst being enterokinase in the first reaction and trypsin in the second reaction.

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## PURIFICATION AND CONCENTRATION OF ENTEROKINASE

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(Accepted for publication, November 11, 1938)

Waldschmidt Leitz (1) has described a method of purification of kinase which consists essentially in drying swine duodenal mucosae with acetone and ether, extracting the dried mucosae with dilute ammonium hydroxide, and removing foreign protein from the aqueous extract by means of dilute acetic acid. The solution can be further purified by precipitation with alcohol, tannic acid, and finally by fractional adsorption on  $Al(OH)_3$  and kaolin. By this method Waldschmidt Leitz obtained a preparation which was about 100 times more active per unit dry weight than the acetone dried mucosa.

This paper describes a simplified method of purification of enterokinase whereby preparations of enterokinase 5,000–50,000 times as active as the acetone dried mucosa can be readily obtained.

The fluid contents of duodena of swine were found to be the most convenient source for enterokinase. The purification consisted principally in pH adjustment and fractional precipitation with ammonium sulfate. The contents of duodena of a large number of freshly killed swine were collected in the slaughter house and stored at about  $-10^{\circ}C$ . The material was afterwards worked up gradually in the laboratory. It has been found that storage of the material frozen as long as a month did not affect its quality as a source of enterokinase.

The details of the process for purification and concentration of the enterokinase are as follows:

1. 2.5 liters of duodenal contents are diluted with 7.5 liters of tap water and warmed to  $20^{\circ}C$ .

2. 50 gm. of "Hyflo Super Cel"<sup>1</sup> are mixed with every liter of mix

<sup>1</sup> Manufactured by Johns Manville Corporation, 22 East 40th Street, New York City.

ture and the whole mass filtered with suction through filter cloth in a 15 inch Buchner funnel First extract

3 Residue is resuspended in 3 liters of tap water and refiltered through cloth

4 Combined extracts are cooled to 5°C

5 pH of extracts is adjusted with 5 N sulfuric acid to about 4.0 (tested with methyl orange) The precipitate formed is filtered rapidly with suction with the aid of 20 gm of "Standard Super-Cel" per liter of solution

6 Filtrate is brought immediately to pH 8.0 with 5 N sodium hydroxide

TABLE I  
*Enterokinase from 2.5 Liters of Duodenal Contents*

	Total enterokinase units [E K U]	Per cent	[E K U] per mg protein nitrogen	[E K U] per mg carbohydrate	[E K U] per mg dry weight
First extract	194,000		100	16	
Washings	69,000		124	20	
Total	263,000	100			
pH 4.0 filtrate	200,000	76	275	19	
Filtrate from 0.4 saturated ammonium sulfate	163,000	62	760	675	40
Acetone dried pig mucosa					0.01

7 Solid ammonium sulfate is added to bring the filtrate to 0.8 saturation The pH of the solution is again adjusted to pH 8.0 with 5 N sodium hydroxide 4.0 ml of 0.4 M pH 9.0 borate buffer is then added to every liter of solution The formed flocculent precipitate is allowed to rise to the surface and is then easily collected into a dough-like mass and removed from the solution Weight of precipitate about 20 gm

8 The precipitate is dissolved in about 5 volumes of cold water and solid ammonium sulfate is added to 0.4 saturation Filtered with suction with the aid of 5 per cent "Standard Super-Cel" Residue rejected

9 Filtrate from 0.4 saturated ammonium sulfate is brought to 0.8 saturation with solid ammonium sulfate and filtered with suction Filter cake about 15 gm

10 Steps 8 and 9 repeated

The degree of purity of the products obtained in the various steps as well as the yields are given in Table I

Table I shows that one fractionation with ammonium sulfate raises the kinase activity of the material by 200 per cent with respect to protein and by 3,000 per cent with respect to carbohydrate

The kinase content per milligram dry weight of the material is about 4,000 times as great as that of acetone dried duodenal mucosa

The material can be further purified by dialysis and by repeated fractionation with ammonium sulfate as well as by fractional precipitation in cold 60 per cent alcohol or cold 2.5 per cent trichloroacetic acid. It was found, however, that the material obtained after the first fractionation with saturated ammonium sulfate was free of any impurities likely to interfere with its use as an activator of trypsinogen

TABLE II  
*Approximate Composition of Purified Enterokinase*

	Per cent
Carbon	45
Total nitrogen	12
Protein precipitable in 2.5 per cent trichloroacetic acid	10 or less
Carbohydrate	10
Glucosamine	10

The filter cake from 0.8 saturated ammonium sulfate is completely soluble in water and thus a solution of any desired strength is readily available

An approximate analysis of the material twice refractionated with ammonium sulfate and then dialyzed is given in Table II. The relative content of protein, carbohydrate, and glucosamine varied considerably in different preparations and no definite conclusion can be drawn as yet as to the actual chemical nature of enterokinase

### *Methods*

*1 Estimation of Enterokinase Activity*—The concentration of enterokinase is expressed in terms of enterokinase units [E.K.U.] per ml. One [E.K.U.] is equivalent approximately to the activity contained in about 100 mg. of acetone dried pigs' duodenal mucosa. The details of procedure of estimation, as well as the definition of the enterokinase unit used are described in the preceding paper (2)

2 *Protein Nitrogen*—The protein nitrogen was determined by the turbidity method (3)

3 *Total Carbohydrate*—Total carbohydrate was determined colorimetrically by the orcinol method of Sørensen and Haugaard (4) and expressed in terms of milligrams of sucrose

4 *Glucosamine*—Adaptation of the colorimetric method of Elson and Morgan (5) The procedure adopted for measuring the glucosamine content of the material used here is as follows 1 ml sample is mixed in a Pyrex 12 × 1.5 cm tube, marked to 10 ml, with 1 ml 2 M hydrochloric acid and heated for 1 hour at 100°C in a steam bath under a reflux condenser about 25 cm long and 6 mm in diameter<sup>2</sup> The solution is cooled, then the following reagents are added 1 drop 0.1 per cent phenolphthalein, 1.0 ml 2 M sodium hydroxide, a few drops of M/1 sodium carbonate to pink color, 1.0 ml of freshly prepared solution of acetyl acetone (0.2 ml in 10 ml M/1 sodium carbonate) The mixture is reheated for 15 minutes at 100°C under the reflux condenser, then cooled 4.0 ml 95 per cent alcohol is added and mixed 1.0 ml of Ehrlich's reagent (0.8 gm *p*-dimethylaminobenzaldehyde in 30 ml 95 per cent alcohol plus 30 ml concentrated hydrochloric acid), and then alcohol to the 10 ml mark are added The solution is carefully but thoroughly mixed and filtered through 9 cm Whatman's No 42 filter paper in order to remove CO<sub>2</sub> bubbles as well as any turbidity from the liquid The color of the solution is compared after 15 minutes with that of a standard glucosamine hydrochloride solution treated in the same manner

The writer was assisted in this work by Margaret R. McDonald and Vivian Kaufman

#### SUMMARY

A concentrated solution of purified enterokinase is conveniently prepared from the fluid contents of pigs' duodena by means of fractional precipitation with ammonium sulfate under the proper pH conditions

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<sup>2</sup> Digestion in M/1 hydrochloric acid longer than 1 hour does not increase the value of the glucosamine readings in the case of this material. Other materials have to be digested longer or in more concentrated hydrochloric acid as found by Palmer, Smyth, and Meyer (6) and also by Sørensen (7)

# THE FLICKER RESPONSE CONTOUR FOR THE ISOPOD ASELLUS

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(Accepted for publication, August 11, 1938)

## I

The form of the curve exhibiting the relation between flash frequency ( $F$ ) and intensity of flash ( $I$ ) critical for response to visual flicker is peculiar in the case of various arthropods previously studied.<sup>1</sup> For sundry vertebrates<sup>2</sup> the curve of  $\log I$  vs  $F$  is either a simple probability integral or else (typically) a composite of two such curves, corresponding to the duplexity of the visual action system.<sup>3</sup> For such forms as bee, dragon fly larva, and fiddler crab,<sup>4</sup> however, the lower portion of the curve increases in slope too rapidly for this formulation. All of these animals have large convex optic surfaces. The deformation of the  $F - \log I$  graph was accordingly attributed<sup>4</sup> to the existence of mechanical conditions affecting reception of light by these eyes. At lower intensities certain of the ommatidia, especially around the periphery of the eye, do not receive sufficient energy to enable them to contribute to the determination of response to flicker within the time permitted by the flash cycle. At higher flash frequencies (and higher critical intensities) the chance of these disadvantaged units being significantly implicated should be much greater, and their effect should therefore be detected. In keeping with expectation, the asymmetry is not affected by changing the temperature over a wide range,<sup>5</sup> but, again as predicted, it is *reduced* by increasing the proportion of light time in the flash cycle.<sup>6</sup>

<sup>1</sup> Cf Crozier, Wolf and Zerrahn Wolf, 1937-38c

Wolf and Zerrahn Wolf 1935-36, Crozier, 1935-36 Crozier, Wolf, and Zerrahn Wolf 1936-37a, d, 1937-38a, d, 1937, 1938c, d 1938a, b, 1938-39, 1937-38b Crozier, 1937 and footnote 15

<sup>2</sup> Crozier, Wolf, and Zerrahn Wolf, 1938a e

<sup>3</sup> Crozier, Wolf, and Zerrahn Wolf 1936-37b 1937-38c, e

<sup>4</sup> Crozier Wolf and Zerrahn Wolf 1937-38c

<sup>5</sup> Crozier, Wolf, and Zerrahn Wolf, 1937-38e



## II

A rather direct test of the validity of these considerations should be obtained by determining the flicker frequency *vs* critical intensity relationship for an arthropod with smaller, flatter eyes—particularly if the ommatidial axes do not diverge too greatly in each eye. The more or less incidental information available with respect to the eyes of isopods<sup>7</sup> indicates that aquatic forms such as *Asellus* should give material for a crucial examination of the point. Our own sections of the eye of *Asellus aquaticus* (histologically a difficult object) show that each eye consists of roughly four rows of rather well separated ommatidia with (save at the edge) approximately parallel axes. Each row shows about 25 ommatidia. It is crudely estimated that each eye is made up of about 125 (or fewer) ommatidia. Its surface is not markedly curved, as in such forms as *Anax*.

The apparatus producing flickered light has been described in our earlier papers<sup>2</sup>. The tests with *Asellus* were made by determining critical intensities at fixed flash frequencies. Observations were made under conditions such that the light time in a flash cycle was equal to the dark time (*i.e.*,  $t_L/t_D = 1$ ). The same type of thermal adaptation and control of temperature (21.5°) was used as in previous experiments with other forms<sup>2</sup>.

The data obtained with *Asellus* show none of the "distorted" asymmetrical features thus far encountered with other arthropods. The  $F - \log I$  curve, like that for the rod-free turtle *Pseudemys*, is a simple, symmetrical function over its whole range. Especially when considered together, these two curves permit a really significant test of the acceptable analytical form of the flicker response contour, because the whole range of the function is open to examination. This has not been possible with vertebrates exhibiting duplexity of visual properties, since only the upper 80 per cent or so of the "cone" curve has been available and the "rod" curve is comparatively minute as well as complicated<sup>2</sup> by its overlapping with the lower (concealed) end of the cone curve.

*Asellus* were kept individually in the cylindrical dishes of thin glass in which their visual responses were tested. To secure precise reactions a good foothold

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<sup>7</sup> Parker, 1891, Peabody, 1938

must be provided. On a glass surface struggling motions may appear at the beginning of a response, and the specific reaction to the moving stripe system can not then be easily recognized. Filter paper accurately cut to fit the bottom of the dish was therefore used to give a surface for easy creeping. *Asellus* is very reactive to vibrations and to disturbance of the water. After being put in place in the apparatus they usually run about for a time. Light may influence this restlessness. During a test the animal must be quiescent when the light is admitted. If it is found to be moving, the diaphragm is closed. Observation is then re begun until the isopod is found to be quiet.

At the threshold reaction to flicker the isopod may dash to the periphery of the jar and then continue crawling in the direction of the movement of the stripes. If it is already at the wall of the container and facing in the direction of the motion of the stripes, the initial response is usually a short dash, which may or may not be continued around the dish. In some cases circus movements are observed in the center of the jar. Frequently recognizable response is obtained when the animal is already in motion, in the form of a sudden change of direction. However, if the isopod is at first facing against the motion of the illuminated stripes the reactions are not clear, the turning movements as result of reaction to the flicker are too slow and not adequately sharp for use as an index, although the critical illumination as then observed is clearly of the same order of magnitude as in the other cases. There is no difference between the critical illumination for response to flicker at any flash frequency, for the non moving *Asellus* whether in the center of the jar or in contact with the wall,—although in the latter instance there is opportunity for a stereotropic factor which might influence the reading.

### III

The data obtained with *Asellus* are given in Table I. The figures are averages obtained from the same 10 individuals at each flash frequency ( $F$ ). The various levels of  $F$  tested were used in practically random order, and there are no detectable drifts from day to day during the course of the experiment. For reasons which have been given in earlier papers,<sup>2-4</sup> the readings were taken in the following way. At each  $F$ , three observations of the critical illumination in a flash are obtained in succession on an individual, the mean of these =  $I_1$  for that animal, the mean value of the  $I_1$ 's for the ten numbered isopods =  $I_m$ , the P. E. of the dispersion of the  $I_1$ 's = P. E.  $I_1$ . These values are (as their logarithms) entered in Table I. Readings are taken at two flash frequencies on each day. In tests made at different  $F$ 's the numbered individuals are used in (different) random orders of succession.

The values of  $I_1$ , for a given  $F$ , can be arranged in an ascending order. The rank-order numbers (1-10) indicating decreasing sequence of excitabilities may then be examined to establish the degree of homogeneity in the group of individuals for which the  $I_1$  readings are averaged. Homogeneity of the group is important for the understanding of the meaning of the data.<sup>8</sup> In the absence of demonstrable homogeneity the data refer to an average performance which may well

TABLE I

Mean critical intensities, and dispersions, at various flash frequencies,  $F$  per sec, for response of *Asellus aquaticus* to flashing light, as  $\log I_m$  and  $\log P E_{1I_1}$  (millilamberts). Light time and dark time equal in the flash cycle ( $t_c, t_d = 1$ ). Temp = 21.5°. The same 10 individuals used at each  $F$ , with three readings on each averaged to give  $I_1$  for each individual,  $P E_{1I_1}$  is the dispersion of these means.

$F$ per sec	$\log I_m$	$\log P E_{1I_1}$
1	$\bar{3} \ 2533$	$\bar{5} \ 5464$
2	$\bar{3} \ 6235$	$\bar{5} \ 9860$
4	$\bar{3} \ 9023$	$\bar{4} \ 2151$
7	$\bar{2} \ 2874$	$\bar{4} \ 8238$
10	$\bar{2} \ 5136$	$\bar{3} \ 1206$
15	$\bar{2} \ 7863$	$\bar{3} \ 0232$
20	$\bar{1} \ 0414$	$\bar{3} \ 4284$
25	$\bar{1} \ 2790$	$\bar{3} \ 6871$
30	$\bar{1} \ 5149$	$\bar{2} \ 0519$
35	$\bar{1} \ 7252$	$\bar{2} \ 0414$
40	$\bar{1} \ 9939$	$\bar{2} \ 5205$
42.5	0 2185	$\bar{2} \ 8005$
45	0 4442	$\bar{2} \ 7317$
47.5	0 6700	$\bar{2} \ 8562$
50	1 0871	$\bar{1} \ 4749$
51	1 5331	$\bar{1} \ 8689$

have no ascertainable meaning. A homogeneous group is one in which the individuals are nearly enough alike for the purposes of the experiment so that they are demonstrably interchangeable. This must be established from the data. This kind of demonstration is curiously absent in studies of the sensory performance of human subjects. Unless it can be established, merely increasing the number

<sup>8</sup> Crozier, 1935-36, 1936

of individuals in the tested group serves only to obscure more completely the nature of the measured phenomenon. At the same time, the discovery in the data of minor persisting heterogeneities can serve to prove the adequate refinement and precision of the methods of observation. It is really impossible to influence subjectively the observations, under the conditions of these experiments, in such a way as to produce accidentally specific correlations of the kinds which the readings disclose.

The mean rank-order numbers for our ten *Asellus* are randomly distributed in time, and show no correlation in pairs of tests made successively on the same individual. The procedure is delicate enough to detect this effect when it is real (*cf.* *Anax* data,<sup>9</sup> turtles<sup>10</sup>). The two individuals whose mean rank-order numbers depart most widely on either side of the mean are Nos 5 and 8. For these the respective mean rank-order numbers with their P E<sub>m</sub>'s are  $7.09 \pm 0.769$  and  $3.84 \pm 0.648$ . The difference is 3.8 times its probable error, and is thus barely on the verge of possible significance. This is borne out by the fact that the mean critical intensities for No 8 are on the average (12 cases out of 16) slightly greater (by ca 4 per cent) than those for No 5 determined at the same time. The evidence of consistency is of interest as reinforcing the indications of the rank-order analysis, and as objectively showing the reliability of the observational procedures employed. This is the general result in all the investigations we have made, with various forms, when an essentially homogeneous population has been available. When *Asellus* Nos 5 and 8 are excluded, the relative rank order positions of the remaining 8 are distributed in an entirely random way. The slight but persisting individualities of Nos 5 or 8 have no detectable influence on the form of the curve which the data give, and the group of 10 is accordingly treated as, from a practical standpoint, homogeneous.

The variation of  $I_1$ , measured by P E<sub>1</sub>, obeys the rule observed with other forms. P E<sub>1</sub> is directly proportional to  $I_m$  (Fig 1, data in Table I). As with other invertebrates for which flicker response

<sup>9</sup> Crozier, Wolf, and Zerrahn Wolf 1936-37b & 1937-38c &

<sup>10</sup> Crozier, Wolf, and Zerrahn Wolf, 1938-39

data have been obtained,<sup>1, 4, 11</sup> there is no "break" in the plot of  $P E_{I_1}$  vs  $I_m$ . This is also observed for the measurements with the turtle *Pseudemys*,<sup>10</sup> in which one encounters the participation of but a single class of neural elements in the determination of the response,<sup>10, 12</sup> for other vertebrates the visual duplexity phenomenon seems to be definitely correlated with a second, different proportionality factor in the variation function for the exclusively cone range of the flicker

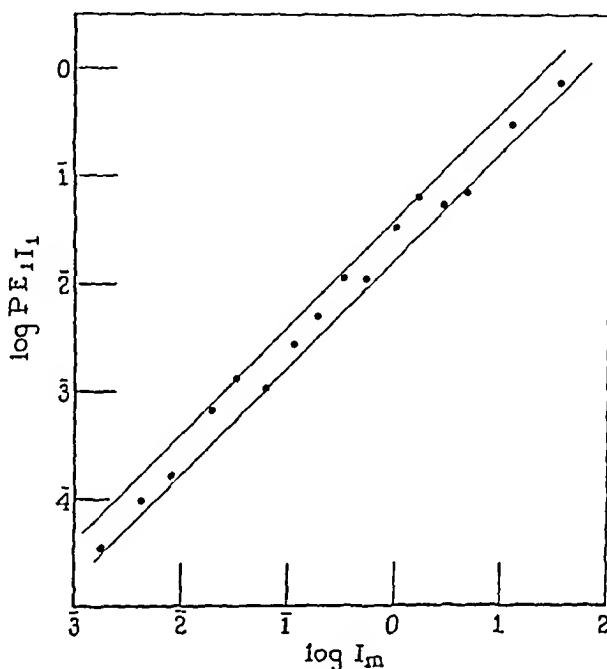


FIG 1 The variation of mean critical intensities ( $I_1$ ) for response to flicker in *Asellus*, as a function of the average critical intensity. The slope of the band = 1, and  $P E_{I_1}$  is consequently directly proportional to  $I_m$ . Data in Table I

response contour.<sup>2</sup> The proportionality factor for *Asellus* is the same as for *Anax*.<sup>13</sup>

#### IV

The relation between  $I_m$  and  $F$  is shown graphically in Fig 2. The curve drawn is a calculated probability integral,

<sup>11</sup> Crozier, 1935, 1935-36

<sup>12</sup> Crozier, Wolf, and Zerrahn-Wolf, 1938a

<sup>13</sup> Crozier, Wolf, and Zerrahn-Wolf, 1937-38c, e

$$F = KP_m \int_{-\infty}^{\log I} \exp \left[ \left( -\log \frac{I}{I_0} \right)^2 / \sigma_{\log I}^2 \right] d \log I, \quad (1)$$

where  $I_0$  is the intensity at the inflection point ( $\log I_0 = \bar{1} 295$ ). The graph differs strikingly from those obtained for *Apis*, *Anax*, and the like, as we have already indicated, the curves of  $\log I_m$  vs  $F$  for the latter are definitely asymmetrical. On the basis of expectation derived from the shape and structure of the eye of *Asellus* we were led to believe that the curve for the latter would not be skewed. The excellent description of the data by a log probability integral is

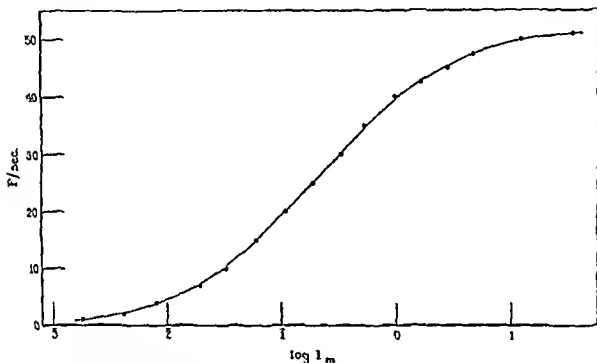


FIG 2  $\log I_m$  vs  $F$  for *Asellus*. Data in Table I. The curve is a calculated probability integral (cf Fig 3).

indicated even better by Fig 3 than in Fig 2. In Fig 3 the extent of the distortion of the flicker response contour for arthropods with large convex eyes is apparent.

The *Asellus* curve is clearly of the same nature as the uncomplicated curve given by the cone retina turtle *Pseudemys*<sup>14</sup>. That it is in each case so well described by a log probability integral is of importance for the theory of the flicker recognition function. The interpretation of this function, of course, cannot in any case be based merely upon

<sup>14</sup> Crozier, Wolf, and Zerrahn Wolf, 1938a, b, 1938-39.

the results of descriptive curve fitting. But in any event until very recently there were not available uncomplicated flicker response contours sufficiently complete in range to provide suitable material for such tests. The curve for *Pseudemys*<sup>14</sup> and that for *Asellus* are free

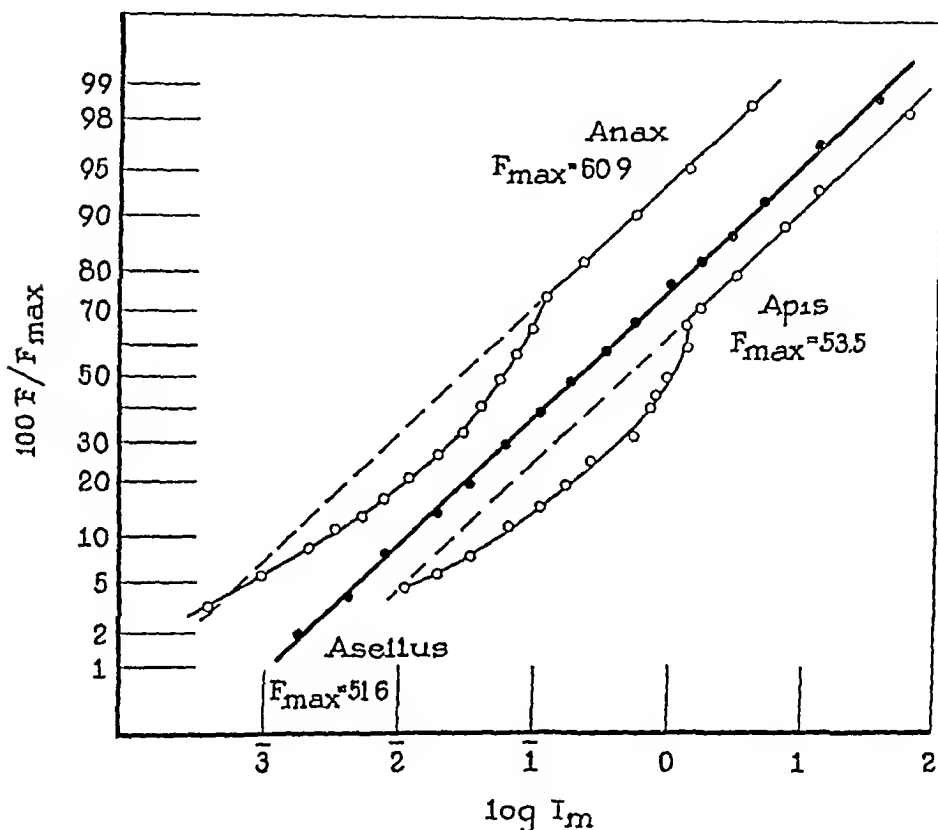


FIG 3 The flicker response contours ( $F - \log I_m$ ) for *Apis*, *Anax*, and *Asellus*, on a probability grid (with  $F$  as a percentage of the respective  $F_{max}$  in each case). The asymmetrical character of the graphs for bee and dragon fly nymph is discussed in the text. The slopes of the three probability integrals (straight lines) are sensibly identical, indicating that  $\sigma'_{\log I}$  is the same for these 3 arthropods, see text.

from structural complications. Each is decidedly well fitted by a probability integral in  $\log I$ . They cannot be adequately fitted by the logistic

$$F = F_{max} / (1 + e^{-s \log I}) \quad (2)$$

Fig 4 shows that a maximum  $F$  may be assumed (about 4 per cent higher than that used for Figs 2 and 3), which will give an apparently

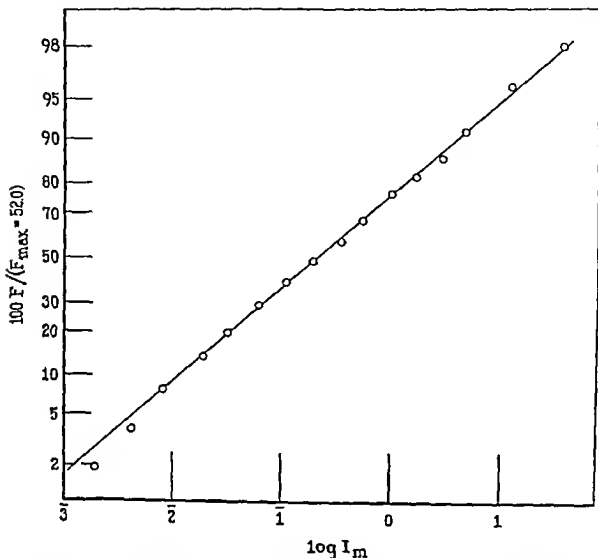


FIG 4 The data of Table I and Fig 2 on a logistic grid. The maximum of  $F$  has been chosen to give nearest approach to rectilinearity. The lower portion of the curve departs widely. Data from experiments on the turtle *Pseudemys*<sup>10</sup> show the same type of departure, and in each case calculation shows that the data on this grid really follow a sigmoid course; this could not be decided with certainty if the upper portion of the curve ( $F \approx 10$  to 50) were the only portion available, see text.

reasonable fit to the upper 80 per cent of the flash frequency range of the data. The fit decidedly fails, however, for the lower end.

Equation (2) is of interest because it is identical with the equation proposed by Hecht<sup>15</sup> for the flicker curve on the basis that  $I$  is pro

<sup>15</sup> Hecht 1937. Hecht, Schlaer, and Smith 1935, Hecht and Smith, 1935-36.



portional to the concentration of photoproducts in the retinal photorensory state

$$pKI = (F)^n / (F_{max} - F)^m, \quad (3)$$

with  $m = n$ ,  $p$  is the proportion of the flash cycle time occupied by light of intensity  $I$ . If (3) is to be used, (2) can be adequate only when  $m = n = 1/2$ . In this event the slope  $d \log F / d \log I$  approaches  $1/2$  as a limit for very low values of  $F$ . If the data do not extend below  $F = 10$  or so, it is impossible to secure an unequivocal graphical adjustment of equation (3) with small integral values of  $m$  and  $n$  ( $1/2$ , 1 or 2). For the two instances (*Pseudemys*, *Asellus*) in which data are reasonably complete this is impossible in any case, since the lower fifth of the curve departs too widely, for the *upper part* a fairly close fit could be gotten with  $m = n = 1$  for *Asellus* and  $= 2$  for *Pseudemys*, but calculation from the data shows that the departures are systematic and significant. Equation (3) also gives erroneous predictions as to the effects obtained by altering temperature<sup>16</sup> and  $p$  the fractional light time in the flash cycle<sup>17</sup>. There is accordingly no real basis whatever for the use of equation (3).

In using the probability integral for purposes of formulation,<sup>18</sup> however, we find a satisfactory fit over the whole range of the data in uncomplicated instances<sup>14</sup> (Fig 2, *etc*). A number of analytical advantages also arise in a simple way in dealing with the duplex (or even triplex<sup>3</sup>) flicker contours for typical vertebrates.<sup>2</sup> The basis for decision in the choice of a descriptive analytical function must, of course, be found in the properties of the parameters of the function. For the probability integral these parameters are (1) the asymptotic maximum ordinate ( $F_{max}$ ), (2) the abscissa of the inflection point ( $\log I_0$ ), and (3) the standard deviation of the first derivative of the curve ( $\sigma_{\log I}$ ). For convenience<sup>18</sup> we write  $\tau'$  for  $\sigma'_{\log I}$ , where the  $\sigma'$  refers to the parameter when  $F_{max}$  is taken as 100 per cent. The changes in the three parameters which result from alterations of retinal area,<sup>19</sup> temperature,<sup>9</sup> and proportion of light time in the flash

<sup>16</sup> Crozier, Wolf, and Zerrahn-Wolf, 1936-37*b*, *c*, 1938*b*

<sup>17</sup> Crozier, Wolf, and Zerrahn-Wolf, 1937-38*d*, *e*

<sup>18</sup> Crozier, 1937

<sup>19</sup> Crozier, Wolf, and Zerrahn-Wolf, 1937-38*c*

cycle<sup>13</sup> show that each of these quantities may be influenced independently of the other two, and in a quantitatively significant way in each case. The behavior of these constants, particularly of  $F_{max}$  and of  $\sigma_{\log I}$ , in interspecific breeding experiments with fishes<sup>20</sup> shows that a definite physical basis must be presumed for each of them in the neural organization of the animal.

The data on several arthropods are of interest for this analysis. The curious and unexpected fact emerges that the value of  $\sigma'_{\log I}$  is nearly or even quite the same for *Apis*, *Anax*, and *Asellus*. This is easily judged by the parallel graphs in Fig. 3. It is to be recalled that  $\sigma'$  is experimentally an invariant, since neither change of temperature, of area, nor of  $t_L/t_D$  appears to affect it detectably.<sup>16, 17</sup> On the other hand it appears to be a characteristic, specific, constant, among vertebrates, since it behaves in a unitary manner in inheritance.<sup>9</sup> It must therefore be determined by the organization of the animal.

The values of  $\log I$  and of  $F_{max}$  are specific for each of the three arthropods tested, but  $\sigma'$  is the same for all. Thus (all at  $t^0 = 21.5$ ,  $t_L/t_D = 1$ ) the values of  $F_{max}$  are *Anax*, 61.0, *Apis*, 53.5, *Asellus*, 51.3, of  $\log I$ , in the same order, 2.45, 1.67, 1.30. It must be concluded that some fundamental feature of neural organization is common to the three. It need not be supposed that the retinal structure supplies this common element. It may well be that the study of other arthropods will show divergent values of  $\sigma'$ . Otherwise one is left with the problem of accounting for the fact that the relative spread of the frequency distribution of intensity thresholds for the elements of effect concerned in the reaction to flicker is identical in bee, dragon fly nymph, and isopod.

## V

### SUMMARY

The flicker response contour for the isopod *Asellus* is a simple probability integral ( $F - \log I$ ) over the whole determinable range ( $F = 1$  to 51). This contrasts with the "distorted" asymmetrical curves obtained with *Apis*, *Anax*, and other arthropods with large

<sup>9</sup> Crozier, Wolf, and Zerrahn Wolf, 1937-1938a, 1938c, and further experiments about to be published.

convex eyes The explanation of the distortion as due to mechanical conditions affecting photoreception is therefore confirmed, as the structure of the *Asellus* eye does not make such a factor likely to be expected for this case

The *Asellus* curve agrees with the only other available complete and uncomplicated flicker response contour (from *Pseudemys*, turtle with rod-free retina), in showing the superiority of the probability integral formulation as compared with certain others which have been suggested

It is noted as a curious and probably important fact that the relative dispersion of the intensity thresholds ( $\sigma'_{log I}$ ) for the elements implicated in determining the flicker contour appears to be identical in bee, dragon fly nymph, and isopod Other relevant information derived from similar experiments with vertebrates shows that this quantity is specifically determined by the organization of the animal The nature of the common feature of neural organization in three such diverse arthropods, as contrasted with the diversity seen within one class of vertebrates (*e g* , teleosts), remains to be discovered

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# THE FLICKER RESPONSE CONTOURS FOR GENETICALLY RELATED FISHES II\*

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## I

A primary condition of rational physiological analysis is the discovery of organic invariants. An organic invariant is a property of the organism expressing its capacity to exhibit a particular kind of performance. The property clearly must be determinate,<sup>1</sup> in the sense that it is experimentally recoverable in adequate repetitions of the observations, else we have no right to regard it as expressing anything. The quantitative measure of this property should be independent of the specific magnitudes of the inciting conditions. Considerations of this sort are peculiarly important for the study of behavior,<sup>1</sup> although obviously not confined to this field, and for the use of data based upon organic response.

Response to visual flicker supplies an example. The elementary problem is to deduce from the properties of the data of such responses the nature of the system in which these properties are determined and by which their exhibition is governed. This is essentially a problem of statistical mechanics. Its solution requires the use of

\* Part I is in *J Gen Physiol*, 1937-38 21, 17-56. Some of the results have been discussed in a series of notes under the title "Specific constants for visual excitation," in *Proc Nat Acad Sc*, 1937, 23, 516, 1938, 24, 221, 542, 1939, 25, 78.

We are under obligation to Dr Gertrud Zerrahn Wolf for assistance in connection with the experiments.

<sup>1</sup> Crozier W J in Murchison, C, *The foundations of experimental psychology*, Clark University Press, Worcester 1929, pp 45-127. 1935, *Déterminisme et variabilité* Paris Hermann et Cie 57 pp.

Crozier, W J, and Pincus, G, *J Gen Physiol*, 1929-30 13, 57, 81. 1931-32, 15, 437. 1936-37, 20, 111.

those invariant indices which the data may be shown to provide. It cannot be directly resolved by qualitative appeal to structural features of the receptor mechanism, for several reasons: the data are basically derived from reactions of the whole organism, and are signals of marginal response to (recognition of) flicker, and the invariant general properties of the data, provided by the type of analytical function describing them, show no specific correlations with known details of structure in different animals<sup>3</sup>. The assumption that visual data are determined by quantitative properties of the retina is unnecessary and probably fallacious<sup>4</sup>. The parallelism with known retinal properties derives simply from the fact that a large number of (variable) elements are involved in each of the two cases, the invariants concerned are essentially statistical quantities.

It is obvious, however, that the flicker response curves for different kinds of animals are not the same. This means that if the same analytical function is usable for all, specific magnitudes must be exhibited by the parameters of the function. To demonstrate the descriptive validity of an interpretive function we require evidence (1) that the curve is in particular cases quantitatively repeatable, (2) that its specific parameters are definitely determined by the constitution of the organism, (3) that the proposed parameters have, experimentally, those physical properties called for by the interpretation.

For a considerable diversity of animals now tested, the law defining the relation between flash frequency ( $F$ ) and flash intensity ( $I$ ) critical for response to flicker is of the same general form<sup>5</sup>. It is of particular moment to supply proof that the *specific* properties of the curve, as distinct from its general character, are determined by the molecular constitution of the organism. This is an essential aspect of the proof that the descriptive invariant indices sought are *organic* invariants—properties of the material considered. This kind of proof can be provided by fortunately chosen cases open to genetic manipulation.

<sup>3</sup> Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., *Proc. Nat. Acad. Sc.*, 1937, 23, 516, 1938, 24, 125, etc.

<sup>4</sup> Cf. Crozier, W. J., and Holway, A. H., *J. Gen. Physiol.*, 1938-39, 22, 341.

<sup>5</sup> *J. Gen. Physiol.*, 1937-38, 21, 223, etc.

The logic of the situation is eminently simple<sup>6</sup> For animals of a given type the curve of (mean) critical illumination ( $I_m$ ) as a function of flash frequency ( $F$ ) with flash cycle of a fixed type, and temperature constant, is a definitely recoverable function The "curve" is of course a band, defined by  $I_m \pm \sigma_I$  as a function of  $F$ ,<sup>7</sup> describing the probability of occurrence of the index response under the given conditions The conditions of observation have been the same in all our experiments, and the variation involved is essentially a constitutional property<sup>8</sup> of the particular organisms concerned in the experiment We need not for present purposes discuss how it may be supposed that this variation in excitability is implicated in, or dependent upon, the mechanism of response We are concerned only, at the moment, with the fact of the reproducibility of the  $F - I_m$  curve, with material of the same type, and with the conjoined fact that the  $F - I_m$  curves for different types of animals, under the same conditions, are not the same

The most useful analytical function adequately describing the dependence of  $I_m$  upon  $F$  (or of  $I_m$  upon  $I$ ) is a probability integral in  $\log I$ <sup>9</sup> The equation for this curve accurately describes the course of the data in cases where only a single assemblage of sensory units is involved, uncomplicated by gross mechanical conditions,<sup>10</sup> and it permits precise dissection of the duplex flicker response contour typical of most vertebrates<sup>11</sup> The three parameters of the probability curve (maximum, abscissa of inflection, and standard deviation of the first derivative) are severally influenced by temperature,<sup>12</sup> retinal area,<sup>13</sup> and fractional light time in the flash cycle<sup>14</sup> in ways which are

<sup>6</sup> *J Gen Physiol*, 1929-30, 13, 57 1936-37, 20, 111, 1937-38, 21, 17

<sup>7</sup> *J Gen Physiol* 1935-36 19, 503 *Proc Nat Acad Sc*, 1936 22, 412  
*J Gen Physiol*, 1936-37, 20, 211, 363 *Proc Nat Acad Sc* 1938, 24, 130  
*J Gen Physiol*, 1937-38, 21, 17, etc

<sup>8</sup> *J Gen Physiol* 1936-37, 20, 211, 363 1937-38 21, 17

<sup>9</sup> *Proc Nat Acad Sc*, 1937, 23, 71, 516, 1938, 24, 125 216 *J Gen Physiol* 1938-39, 22 311, 451 in press

<sup>10</sup> *J Gen Physiol* 1938-39 22, 311 451 in press

<sup>11</sup> *J Gen Physiol*, 1937-38 21, 17 203 313 *J Exp Zool*, 1939, in press

<sup>12</sup> *J Gen Physiol*, 1936-37, 20, 393 411 *Proc Nat Acad Sc* 1938, 24, 216

<sup>13</sup> *J Gen Physiol*, 1937-38, 21, 223

<sup>14</sup> *J Gen Physiol*, 1937-38, 21, 313, 463

predictable by the conception of variable excitation units if these three parameters are acceptable as sufficient and efficient for the analytical function describing the data. This is sufficiently demonstrated by the fact that situations superficially dissimilar but in essential respects dynamically identical (*e g*, auditory excitation, nerve excitation, and photosynthesis) are describable by the same formulation<sup>15</sup>. No other available descriptive function adequately fits the measurements, although several have been proposed,<sup>16</sup> nor do the constants which these alternative descriptions contain exhibit the requisite physical properties which theory and experiment alike demand,<sup>17</sup> they therefore do not need to be considered.

The problem arises therefore as to whether the specific magnitudes of the parameters of the probability integral can by a genetic test of their constitutional dependence be shown to exhibit the behavior of natural constants. In the biological situation, and for the present problem in particular, this of course means *specific* constancy correlated with constitution. This can be tested by breeding experiments. For this purpose fresh water teleosts are peculiarly advantageous. Various genera which we have examined give different  $F - \log I_m$  curves. Several "species" and forms of each of several different generic types show agreements in this respect, as we have already found indicated.<sup>1</sup> The constitutional differences involved in the control of the  $F - \log I$  curves therefore must be dependent upon rather deep-seated features of organization. Different genera, with certain limitations, can be successfully hybridized, as is well known.

We shall distinguish the three parameters of the probability function by symbols— $F_{max}$ , the maximum flash frequency to which the curve rises,  $\tau'$ , the  $(\log I)$  abscissa of the inflection point, and  $\sigma'_{\log I}$ , the standard deviation of the first derivative of  $F$  vs  $\log I$ ,

<sup>15</sup> *Proc Nat Acad Sc*, 1937, 23, 71

<sup>16</sup> Hecht, S, and Verrijp, C D, *J Gen Physiol*, 1933, 17, 251. Hecht, S, Shlaer, S, and Smith, E L, Intermittent light stimulation and the duplicity theory of vision, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1935, 3, 237. Hecht, S, *Physiol Rev*, 1937, 17, 239. Hecht, S, *The Harvey Lectures*, 1937-38, 33, 35.

<sup>17</sup> *J Gen Physiol*, 1936-37, 20, 393, 411. *Proc Nat Acad Sc*, 1938, 24, 216. *J Gen Physiol*, 1937-38, 21, 313, 463.

with  $F_{max} = 100$  per cent. It is to be expected that in different forms  $\tau'$ , and  $F_{max}$ , with temperature and  $t_L/t_D$  constant, may well be influenced by complex circumstances affecting (a) the total number of potentially excitable elements, and (b) the basic excitability of each (any) element. This is based upon the experimentally determined influence of temperature<sup>12</sup> and flash cycle light time fraction<sup>14</sup>. But  $\sigma'_{log I}$  could be expected to be a more direct expression of the fundamental molecular organization of the animal, since it measures the relative dispersion of the  $\log I$  thresholds of the relevant population of neural elements, this quantity is experimentally independent of temperature<sup>12</sup> and of the light time proportion in the flash cycle,<sup>14</sup> and of retinal area,<sup>13</sup> but it is a specific property already indicated to be rather simply determined genetically<sup>18</sup>. (The situation may in this respect be complicated by certain purely mechanical factors among arthropods utilized for similar experiments,<sup>19</sup> but this does not really affect the immediate argument.)

In dealing with the flicker response contours of most vertebrates we encounter a situation which has a number of interesting features. Two populations of excitable elements are usually involved in the exhibition of the response to flicker, as revealed by the form of the flicker response contour. One of these has a modal excitability at a rather low critical intensity, the other (larger) has a modal excitability at a much higher intensity.<sup>20</sup> In terms of the duplexity theory these have been interpreted as due to the excitation of retinal rods and cones respectively. There are curious pitfalls in the logic of this proposition, into the discussion of which we need not now go. We are concerned only with the objective evidence that two populations of neural effects are concerned. In a genetic experiment with fishes<sup>18</sup> evidence has been provided that these two populations may in hereditary determination follow different rules. The properties of the flicker response contour in single types of individuals therefore provided important instances of the significance of the viewpoint here developed for the analysis of functional properties.

<sup>12</sup> *J Gen Physiol*, 1937-38, 21, 17. *Proc Nat Acad Sc*, 1937, 23, 516.

<sup>13</sup> *J Gen Physiol* 1937-38, 21, 223. 1938-39, 22, 451.

<sup>14</sup> Cf Hecht S. *Physiol Rev*, 1937, 17, 239. *J Gen Physiol*, 1936-37, 20, 211. 1937-38, 21, 17. 203. *J Exp Zool*, 1939, in press.



Our first test of this matter<sup>18</sup> was exploratory. We used a back-cross stock derived from *Xiphophorus helleri* ( $X$ )  $\times$  *Platypoecilus maculatus* ( $P$ ),  $F_1$ 's being mated to  $X$  *helleri*. These individuals ( $H'$ ) were uniform in their flicker response contours (i.e., showed no evidence of segregation into several types). There was indicated a fairly simple relationship between the specific values of the parameters of the hybrid curve and of the curves for the two parental stocks. The differences found between various genera of fishes have to do with  $F_{max}$ ,  $\log I_{infl}$  ( $= \tau'$ ), and  $\sigma'_{\log I}$ , for the "rod" and "cone" portions of each duplex curve, and with the separation between  $\tau'$  for the two parts. There is no evidence of formal association between magnitudes of these parameters in our data on various vertebrates, nor with respect to the rod and cone curves for any one kind of animal. (This refers, of course, to data obtained at one temperature, and with a flash cycle in which  $t_L = t_D$ , we do not as yet know in sufficient detail just how the temperature characteristics for  $\tau'$ , and the proportionality factors for change of  $F_{max}$  and  $\tau'$  with change of percentage light time, may also show specific association with genetic types.) In the breeding experiment<sup>18</sup> referred to it appeared that the cone  $F_{max}$  in the hybrids was the same as for  $X$ , and the values of  $\sigma'_{\log I}$  for both rod and cone curves, for the rod part, however,  $F_{max}$  was the same as for  $P$ ,  $\tau'$  for both rods and cones was intermediate between the values for  $X$  and  $P$ , but closer to those for  $P$ .

Scrutiny of these findings shows that dominance with respect to one aspect of the flicker contour may be accompanied by "blending" with respect to another, and the dominance relations may be quite different in the two parts of the duplex curve. These are sufficient illustrations of the necessity for knowledge of the complete function before its rational interpretation and use become possible. Unquestionably many another genetic situation will in time be fruitfully explored from this standpoint.

It is also clearly indicated that the parameters  $F_{max}$  and particularly  $\sigma'_{\log I}$  behave as if determined by some relatively simple fact of organization. The presumption is encouraged that a determinate physical basis exists of which they are expressions. They refer respectively to the total number of excitation elements, given by

$\int_{-\infty}^{+\infty} dF/d \log I$ , and to the frequency distribution of the  $\log I$  thresholds

The resistance of  $\sigma'_{\log I}$  to experimental conditions, shown in its constancy with respect to area, temperature, and fractional light time in the flash cycle, is greater than that of  $F_{max}$ , which (although independent of temperature) is in simple proportion to the percentage dark time in the flash cycle. It is, of course, probable that the proportionality factor in this relationship would also prove to be a specific characteristic. Conditions might easily be conceived in which the value of  $F_{max}$  (for a given ratio  $t_L/t_D$ ) could be modified by circumstances affecting the excitability cycle of each irritable element, this is in line with the explanation<sup>14</sup> for the direct proportionality of  $F_{max}$  to  $t_D/(t_L + t_D)$ . Hence the genetic behavior of  $F_{max}$  might well be expected to be less simple than that of  $\sigma'_{\log I}$ . The same applies to the abscissa of inflection,  $\tau'$  or  $\log I$ . The dependence of  $\tau'$  upon temperature and  $t_L/t_D$  is simple, and of such a sort as to show that conditions proportionately affecting the excitabilities of all the elements in the same way naturally shift the  $F - \log I$  curve to a new position on the  $\log I$  scale, without changing  $F_{max}$ . Any number of circumstances involving the chemical control of the individual excitabilities, or even conditions modifying the mere optical transmission of the ocular mechanism, could be expected to modify  $\tau'$ . The question as to whether the temperature characteristics for the shift of  $\tau'$  are specific, and heritable,<sup>21</sup> presents another aspect of this matter which will ultimately require investigation, but concerning which we have as yet no information, the corresponding question as to the quantitative dependence of  $\tau'$  upon  $t_L/t_D$  is likewise unexplored.

In developing an interpretive theory of the flicker response contour there are to be recognized two distinct aspects

(a) The proof that the shape of the curve is dependent upon the constitutional organization of the animal is supplied by the facts of reproducibility under fixed conditions, differences with different types of animals, invariance under alteration of environmental factors, and particularly its behavior in inheritance. This provides the conditions under which it becomes possible to undertake a de-

<sup>1</sup> For an analogous instance cf Pincus G *J Gen Physiol* 1930-31, 14, 421

termination of the properties of the curve as revealed by the relations of these properties to experimentally controllable variables

(b) It does not select for us the particular analytical function most appropriate for the data. It does, however, supply explicit criteria which the chosen function must satisfy. The results of this first experiment, as we indicated, were surprising—since no definite, particular expectations of simplicity in the result could have been reasonably entertained. A second experiment of this type was therefore planned, under somewhat simpler conditions. The  $F_1$  progeny of a cross between *Xiphophorus* and *Platypoecilus* was to be examined, and the  $F_1$ 's interbred. We sought to utilize if possible species of  $X$  and  $P$  different from those involved in the earlier test. For assistance in obtaining stocks of the desired animals we are under obligation to our associate Dr C P Haskins. This paper is based on the data secured with  $X$ ,  $P$ , and their  $F_1$  offspring. The results with  $F_2$  will be considered in a subsequent report.

## II

The observational procedure used has been kept as uniform as possible. Groups of individuals are well adapted to routine conditions of feeding and experimental handling. Where possible a lot of 10 is employed. In certain cases a smaller set must be used as no more fishes of that particular type are available. Records are kept for each individual separately. Three successive observations of critical illumination are made on each individual, at each flash frequency. The individual means ( $I_1$ ) of these sets of three are averaged for the group, and this appears as  $I_m$  in the tables. This is done only if analysis of the data shows that the individuals in a group are equivalent. The reasons for this averaging have been considered previously,<sup>18</sup> and the tests for equivalence (homogeneity). The readings of critical illumination are amply sensitive to detect real individual differences even when these are quite small.<sup>22</sup> The succession of the levels of  $F$  tested is so arranged as to reveal drifts of  $I_m$  with time during the completion of a curve, should these occur.

<sup>22</sup> *J Gen Physiol*, 1936-37, 20, 363, 393, 1937-38, 21, 223, 1938-39, 22, 311  
*J Exp Zool*, 1939, in press

The apparatus has been described<sup>18</sup> Temperature was maintained at 21.5° The flash cycle used had equally long intervals of light and no light

The data on *λ montezuma* are given in Table I It is apparent from comparison with the measurements with *X helleri*<sup>18</sup> at the same flash frequencies that the agreement between the results with these

TABLE I

Showing the essential similarity of the flicker response contours for two species of *Xiphophorus* Mean critical intensities with the P.E.'s of their dispersions (milliamperes) Temperature 21.5 C flash cycle with equally long light and dark intervals The data on *λ helleri* are taken from Crozier, Wolf and Zerrahn Wolf *J Gen Physiol*, 1937-38 21, 25 only data at the indicated flash frequencies are repeated here the complete set of measurements is given in Fig 1 (together with data on albino individuals<sup>3</sup>) There is no significant difference between the measurements for *λ montezuma* and *λ helleri* Three observations on each of the same 10 individuals in all cases

F per sec	<i>Xiphophorus montezuma</i>		<i>Xiphophorus helleri</i>	
	log $I_m$	log P.E. $1/2$	log $I_m$	log P.E. $1/2$
3	6.3528	7.0030	6.3420	7.0969
6	5.3354	7.9304	5.3721	7.9653
			5.2826	7.7148
			5.3539	7.8341
9	5.2393	7.7749	5.2469	7.8295
			5.2378	7.8374
15	4.8650	7.4494	4.8918	7.2650
25	4.4639	7.1501	4.4708	7.9719
			4.4598	7.9677
35	4.0172	7.7698	4.01520	7.9002
42	4.3105	7.7176	4.3057	7.5398
			4.2785	7.5864

two superficially quite different animals is remarkably close We have already shown<sup>3</sup> that albino mutants of *λ helleri* give figures not distinguishably different from those gotten with the wild type *λ helleri* The determinations with the three forms of *λ xiphophorus* are plotted together in Fig 1

<sup>3</sup> *Proc Nat Acad Sc*, 1938, 24, 221

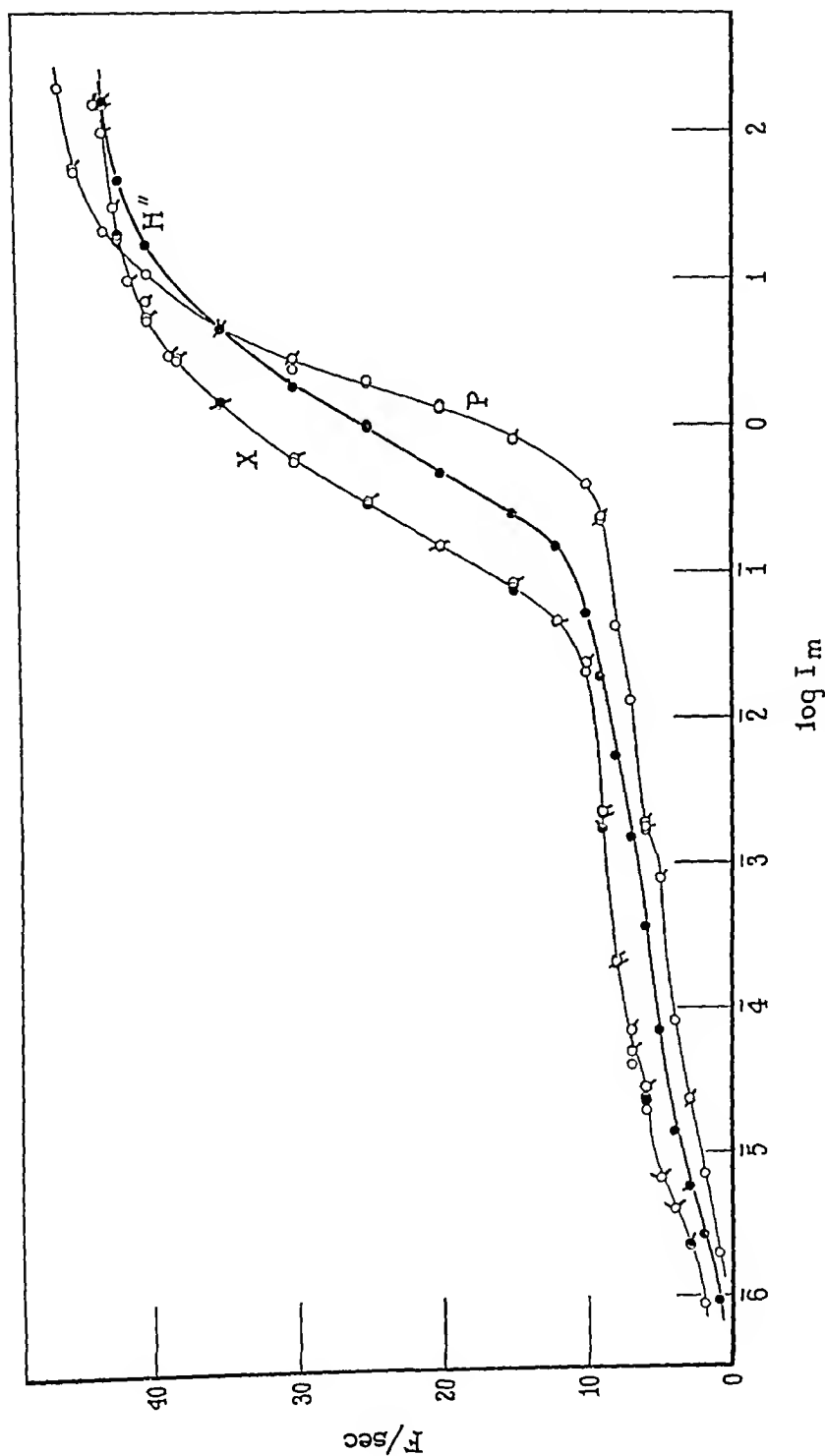


FIG 1 Mean critical intensities as a function of flash frequency ( $t^\circ = 21.5$ ,  $t_L = t_D$ ) for (X) Several types of *Xiphophorus* ( $\bullet = X$  *montezuma*,  $\circ = X *helleri*, with duplicating points as  $\circ'$ ) for which data are given in Table I, and Q = *X* *helleri* var *albino*<sup>23</sup>, P = several types of *Platypoecilus* (Table II,  $\circ = P$  *maculatus*,  $\circ = P$  *variatus*), H'', F<sub>1</sub> hybrids from *X* *montezuma*  $\times$  *P* *variatus*, data in Table III$

Similar agreement of measurements with five different forms of *Platypocilius*—including one species hybrid—is shown in Table II (Fig. 1)

These data agree with those previously secured<sup>18</sup> in showing the essential invariance of the flicker response contour under fixed conditions of temperature and light time fraction, over a considerable interval of time, for each of the generic types. The difference between the two curves, on the other hand, is striking and complex (cf. Fig. 1). The variation of  $I_1$  follows the usual rule for verte

TABLE II

Showing the essential quantitative agreement between the flicker response contours for various forms of *Platypocilius*: *P. maculatus nigra* (10 individuals), *P. variatus* (red tail) (3 individuals), *P. v. nigra* (2 individuals), *P. v. rubra* (2 individuals), *P. v. gold* (5 individuals), hybrids (2 individuals) between *P. variatus rubra* and *P. maculatus*. Three observations on each individual at all points. Temperature 21.5°C, flash cycle with 50 per cent light time. The variation indices are plotted in Fig. 2.

F per sec.	Red tail <i>P. variatus</i> $\log I_m$	<i>P. v. nigra</i> $\log I_m$	<i>P. v. rubra</i> $\log I_m$	Gold platy <i>P. variatus</i> $\log I_m$	Hybrids <i>P. v. rubra</i> $\times P. maculatus$ $\log I_m$
3	5 3583			5 3786	5 3592
6	5 2430 (3 2725)	3 2480	3 2721	5 2290	3 2534
9	1 3526 (1 3608)	1 3535	1 3699	1 3595	1 3570
15	1 8980			1 8846	1 9135
25	0 3002			0 2833	1 3107
35	0 6601			0 6544	0 6815
45	1 7327			1 7278	1 7582

brates with duplex flicker contours, in that  $PE_1$  is directly proportional to  $I_m$  but beyond a certain intensity the proportionality constant changes.<sup>24</sup> This seems to be due to the complete dropping out of effects due to the rod population of elements above this particular intensity.<sup>4</sup> For these genera the break in the variation function (Fig. 2) is at comparatively high flash intensity (ca. antilog 0.5), so that only a small part of the data fall above this level. The mean

<sup>4</sup> *J. Gen. Physiol.* 1935-36, 19, 503. 1936-37, 20, 211. 1937-38, 21, 17, 203. *J. Exp. Zool.*, 1939 in press.

variation with  $P$  is somewhat, and significantly, lower than with  $X$  at the same level of  $I_m$ —for the intensities below antilog 0.5 in the ratio of 1:1.41. Analyses of the  $X$  and  $P$  duplex curves have already been given in some detail<sup>18</sup>. By extrapolation of the cone curve toward  $F = 0$  it is found that the “bump” on the nearly hori-

TABLE III

Mean critical intensities for response to visual flicker, with the P.E. of their dispersions (millilamberts) at various flash cycles for  $F_1$  hybrids between *X. montezuma* and *P. variatus*, three observations on each of the same 3 individuals at all points. Temperature 21.5°C, flash cycle with 50 per cent light time. See Figs. 1 and 2.

$F$	$\log I_m$	$\log P.E./I_1$
1	$\bar{7}.9561$	$\bar{8}.2335$
2	$\bar{6}.4140$	$\bar{8}.3892$
3	$\bar{6}.7543$	$\bar{8}.8189$
	$\bar{6}.7487$	$\bar{8}.3705$
	$\bar{6}.9534$	$\bar{8}.8235$
4	$\bar{6}.1348$	$\bar{7}.4622$
5	$\bar{6}.8390$	$\bar{7}.3373$
6	$\bar{4}.5544$	$\bar{6}.8235$
7	$\bar{3}.1708$	$\bar{5}.4355$
8	$\bar{3}.7273$	$\bar{4}.2953$
9	$\bar{2}.2730$	$\bar{4}.8475$
10	$\bar{2}.7118$	$\bar{3}.0759$
12	$\bar{1}.1706$	$\bar{3}.8098$
15	$\bar{1}.3993$	$\bar{3}.5833$
20	$\bar{1}.6701$	$\bar{2}.0795$
25	$\bar{1}.9880$	$\bar{2}.5750$
	0.0020	$\bar{3}.9943$
30	0.2596	$\bar{2}.9016$
35	0.6767	$\bar{2}.6062$
	0.6751	$\bar{2}.9928$
40	1.2370	$\bar{1}.0430$
42	1.6761	$\bar{1}.7760$
43	2.2063	0.2100

zontal intermediate portion of the curve (cf. Fig. 1) coincides with the start of the cone curve. For this extrapolation a log logistic may be used,<sup>18</sup> but a probability integral<sup>18</sup> is better. Cases in which the whole course of the curve may be followed for a single uncomplicated population of sensory effects<sup>9</sup> permit a decision between these

two equations. The greater separation of duplicate estimations of  $\log I_m$  in the region of beginning of the cone curve as compared with

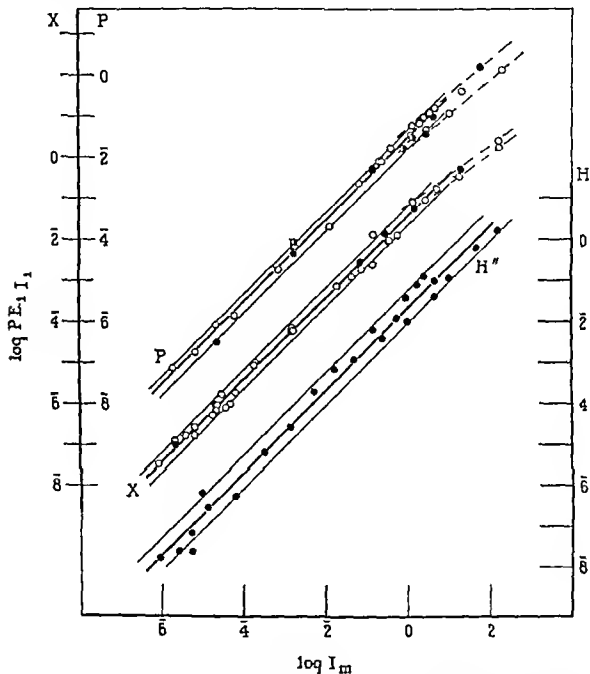


FIG. 2. The relation between dispersions of the measurements of critical intensity and the means, for *Platyopocilius* (*P*),  $\circ = P. maculatus$ ,  $\bullet = P. variatus$ , *Xiphophorus* (*X*),  $\circ = X. helleri$ ,  $\bullet = X. montezuma$  and the  $F_1$  hybrids (*H*). Data in Table I.

other regions of the curve<sup>18</sup> is again confirmed by the new measurements.



Three  $F_1$  hybrids ( $H''$ ) between *X monlezuza* and *P variatus* were used. They were entirely equivalent. Observations were obtained at nineteen flash frequencies. The data are summarized in Table III (see Fig 1). The variation data (Fig 2) show mean values of  $PE_{1,1}$  a little lower than for *X* and *P*, but this is probably a simple consequence of the smaller number of individuals (3) and of

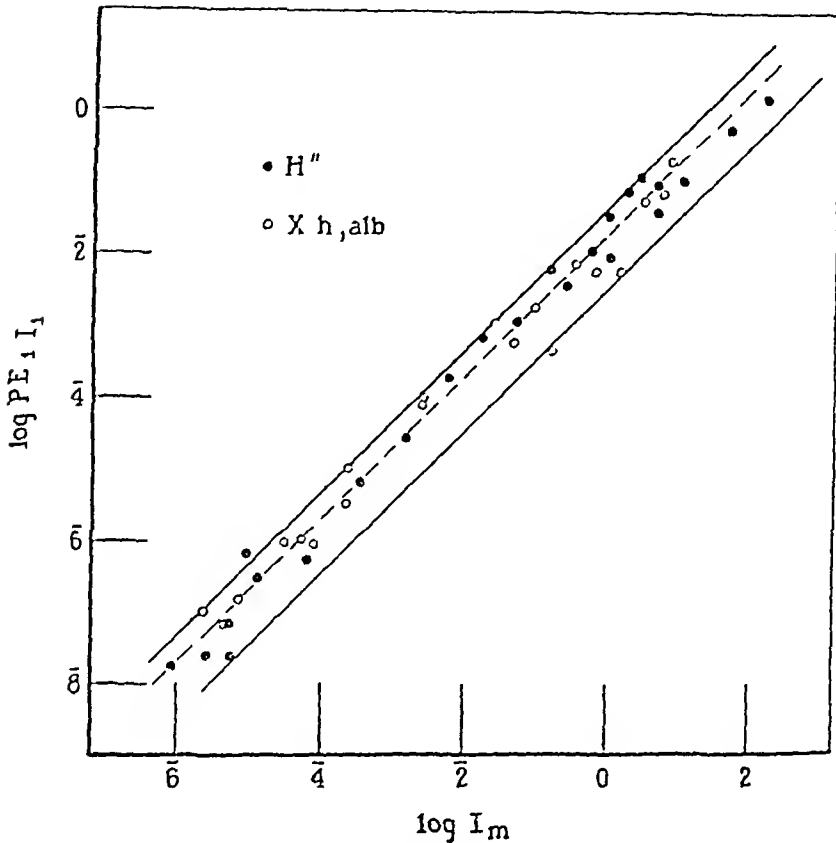


FIG 3 The variation of  $I_1$  follows the same rules for the  $F_1$  hybrids and for a set of the same number of individuals (three) of *X helleri* var *albino*. See text

readings (9) at each point. The variation data for  $H''$  indeed agree precisely with those from *X helleri* var *alb*,<sup>18</sup> where the same numbers were used (Fig 3).

The behavior of these fishes exhibits certain minor differences with respect to the elicitation of the index response<sup>18</sup>. *X monlezuza*, like *helleri*, is very active and is liable to disturbance by vibrations

The response to flicker consists in the beginning of swimming in the direction of motion of the revolving stripes. This may be quickly stopped so that the fish is quite stationary but with rapid fin undulations. Males especially may also show a quick shaking (rolling) motion at the threshold of the response. The reactions used are only really clear when the fish is near the wall of the container and headed in the direction of revolution of the stripes. These conditions sometimes must be patiently waited for. At low  $F$ 's (below 10) the response is as clear as the movement of a galvanometer needle. With higher  $F$ 's the tendency to rigidity is more pronounced. In these matters the hybrids resemble  $X$ , and not  $P$  (*cf* footnote 18), but the shaking movements are less in evidence.

### III

The data of Tables I, II, and III, are plotted in Fig 1. Analysis of the  $H''$  curve by the method already used with other forms<sup>18</sup> exhibits (Fig 4) the dissection of the duplex graph into a lower portion (rod) and an upper (cone) part with a region of overlap in which the effectiveness of the rod contribution progressively declines. The dissection is made by extrapolation of the probability integral adjusted to the upper portion of the  $H''$  data in Fig 1. The declining rod curve is obtained by difference. The reasons for treating  $F$  as additive have been considered previously.<sup>5</sup>

The uncomplicated cone portions of the flicker contour for  $\lambda$  and  $H''$  are given upon a probability grid in Fig 5. The data upon the backcross hybrid  $H'$  of  $\lambda$  *helleri* and  $P$  *maculatus* previously studied<sup>18</sup> are also included. A satisfactory fit is obtained by finding the value of  $F_{max}$  which makes the graph (Fig 5) rectilinear. For  $H'$  and  $X$ ,  $F_{max}$  is essentially identical, respectively 43.00 and 43.05/sec. For  $H''$  a slightly higher value is required, 43.34. But the slope is the same for all three. This of course means that the cone  $\sigma'_{log I}$  is identical. It was shown previously<sup>18</sup> that for  $P$   $F_{max}$  is of course much higher and  $\sigma'_{log I}$  much less.

The abscissa of inflection for  $H'$  and  $H''$  is intermediate between those for  $X$  and  $P$ , that for  $H''$  less ( $I = 0.576$ ) than for  $H'$  ( $I_i = 0.832$ ).

<sup>25</sup> *J. Gen. Physiol.* 1936-37 20, 411

It has been pointed out that  $F_{max}$  could well be influenced by factors tending to increase the effective size of the population of sensory elements. *X montezuma* is larger than either *X helleri* or the hybrids, but there are no differences in any of the fishes we have tested to be correlated with individual differences of size. The effect is probably of a more subtle sort, like that induced by a slight shortening of the light time interval, as already suggested (section I). The agreements of  $\sigma'_{log I}$  are definite and striking. They must be taken as evidence for the dominance of *X* type of cone population over that seen in *P*. The shift of  $\tau'$ , different in the two kinds of hybrids considered, is exactly of the sort produced by a lowering

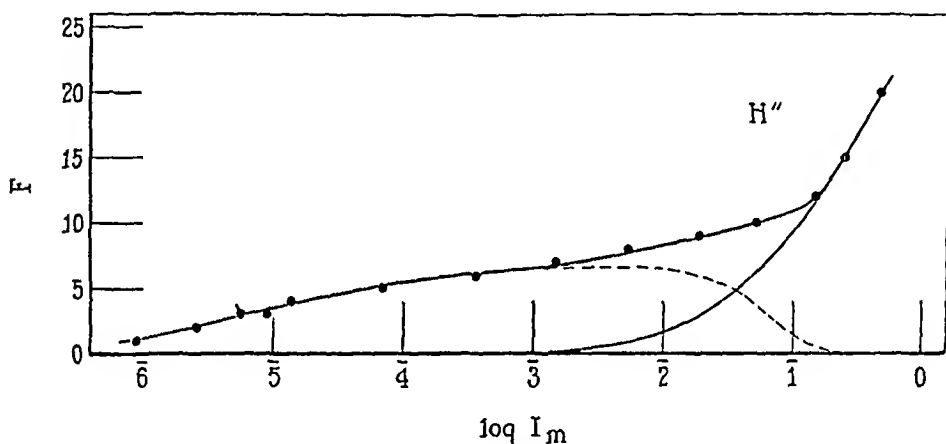


FIG 4 Analytical dissection of the duplex flicker contour for  $H''$  by extrapolation of the probability integral which describes the upper segment of the graph (Figs 1 and 5) See text

of the temperature<sup>12</sup>. It clearly corresponds to the effect predictable if in the hybrids the effective concentration of material governing excitability were to be reduced, so that with temperature fixed the rate of chemical transformations for which it is responsible is lowered—therefore a higher intensity of light is required for response at a fixed  $F$ . (The reasons leading to this type of interpretation have been gone into at some length in other connections<sup>26</sup> and do not require restatement here.) The effect cannot be interpreted as due

<sup>26</sup> *J Gen Physiol*, 1938–39, 22, 311, 487

to a difference in the transparency of the optic media, since the shift for rod and cone segments is not the same (No iris movements are involved either) It would be expected on this basis, that the temperature characteristics for the cone curves  $X$ ,  $H'$ , and  $H''$  should

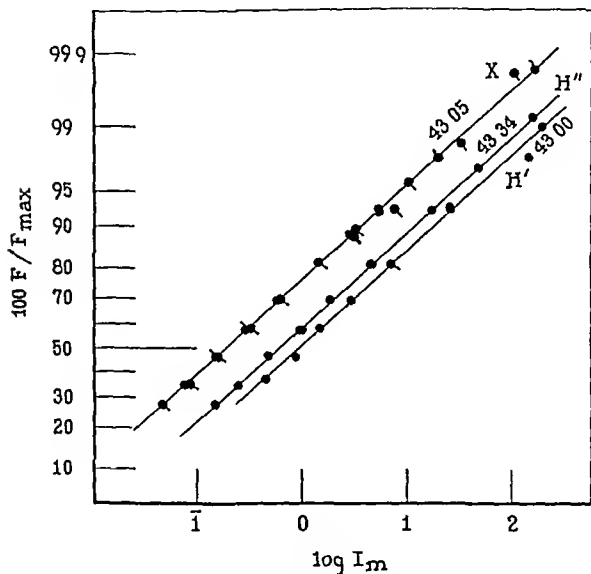


FIG 5 The upper segments of the flicker contours for *Apophor* and two kinds of its hybrids with *Platyopocilus* on a probability grid The slopes are identical (i.e.,  $\sigma'_{t, \lambda}$  is the same) The curves are computed to maxima 43.05 ( $\lambda$ ), 43.34 ( $H''$ ), and 43.00 ( $H'$ )

be the same, although this might be difficult to establish if two temperature characteristics are exhibited over the explorable range of temperatures<sup>6</sup> The rôle of such considerations in suggesting experimental tests of the mechanism involved to account for the phenom

ena connected with critical temperatures<sup>26</sup> is not to be lost sight of, and may easily prove to be quite important

The outcome of the analysis, as regards the cone section of the graph, is to make it fairly evident that slight, quantitative modifications, expressed through the activity of essentially the same kind of population of sensitive elements, with the same type of physico-chemical architecture, must be conceived to determine the form of the upper (cone) segment of the flicker response contour in *Xiphophorus* and in its hybrids with *Platydocilus*. The shape of the curve provides an elementary instance of classical hereditary dominance. It is obvious that from the standpoint customarily used in the majority of genetic analyses this phenomenon could not be recognized (*cf* also footnote 27). It is also clear that with respect to the same kind of organic performance two quite different sorts of hereditary influence are simultaneously manifest.  $\tau'$  supplies an example of "blending,"  $\sigma'_{\log I}$  of simple dominance. This serves as another<sup>27</sup> instance of the way in which situations may be totally obscured in the absence of knowledge of the entire course of the performance contour. The case becomes even more curious and illuminating when the lower (rod) part of the curve is carefully examined.

For  $H'$  it was shown that in the rod region  $\tau'$  is also intermediate between that for  $X$  and for  $P$ . This is likewise true for  $H''$  (Fig 6).  $F_{max}$  (rods) for  $H'$  was, however, equivalent to that for  $P$  although  $\sigma'_{\log I}$  was that of  $X$ . With  $H''$  it is apparent that  $F_{max}$  is exactly intermediate between those for  $X$  and for  $P$ , but  $\sigma'_{\log I}$  is identical with that for  $P$  (Fig 6).

The fuller interpretation of these facts depends in great measure on the outcome of tests made with  $F_2$  and backcross stocks which (with certain limitations due to the genetic nature of generic crosses) should give evidence as to possible segregation phenomena. This information we expect to be able to provide in the near future. It is made reasonably evident, however, that there are indeed involved in the flicker contour the properties of two distinct groups (populations) of sensory effects<sup>28</sup>. Whether these two are to be properly correlated in a direct way with the attributes of retinal rods and cones

<sup>27</sup> *Cf J Gen Physiol*, 1936-37, 20, 111

<sup>28</sup> *Proc Nat Acad Sc*, 1938, 24, 125. *J Gen Physiol*, 1937-38, 21, 17

is a question beset with perplexities of a kind usually overlooked, and upon which we need not for present purposes elaborate. The two groups of effects are manifestly governed by different processes, since their determinants do not obey the same rules in inheritance. This is perfectly consistent with the fact that the relations of the rod and cone segments to temperature are not quite the same, in the one case thus far investigated,<sup>9</sup> the fact that the temperature characteristics<sup>9</sup> and the effects of supranormal temperatures<sup>30</sup> are the same for both merely shows that the character of the essential physico

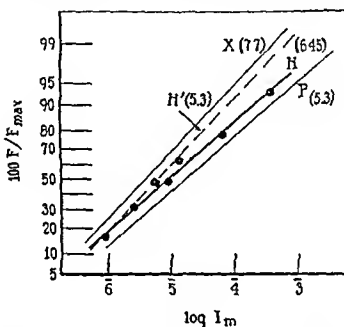


FIG. 6 The initial rising segment of the flicker response contour for the  $\bar{I}_1$  hybrids ( $H''$ ) produced by  $\lambda \times P$  has the slope constant ( $\sigma'_{I, I}$ ) of  $P$  the cross bred progeny  $H'$  produced in another type of experiment have  $\sigma'_{I, I}$  equivalent to that for  $\lambda$ . The respective values of  $F_m$  are  $\lambda$  77  $P$  53,  $H'$  53,  $H'$  645. See text. The lines for  $X$ ,  $P$  and  $H'$  are taken from the analyses previously published.<sup>18</sup>

chemical organization in the two sets of elements is the same,<sup>31</sup> it does not imply that the amounts of governing substances in the excitability systems of the two sets are identical. The differences

<sup>9</sup> *J. Gen. Physiol.*, 1938-39, 22, 487.

<sup>30</sup> *J. Exp. Zool.*, 1939, in press.

<sup>31</sup> Also shown by the fact that in the same species the proportionality constants for enlargement of the cone and rod segments of the curve as the light time fraction in the flash cycle is increased, are identical. *J. Gen. Physiol.*, 1937-38, 21, 313.

in the behavior of rod and cone sections of the curve with respect to dominance relations, as in  $H''$   $\sigma'_{log I}$  for one follows  $P$  and the other the  $X$  parent, complement the state of affairs in  $H'$ , where each adheres to the  $X$  type, and rounds out the proof that the hereditary determination of the rod section is independent of that for the cone part

## IV

The curve obtained by subtraction of the backward extrapolation of the cone probability integral from the composite flicker contour yields the difference curve<sup>18</sup> held to depict the way in which the dropping out of the rod contribution depends upon intensity as the excitation of the cone population increases. Fig 6 has given this curve for  $H''$ . The statistical conception of the excitation elements required for the analysis<sup>32</sup> suggests that this curve should also be described by a probability integral. This has been demonstrated in various instances<sup>11</sup>. Fig 7 shows that the same principle applies for the data on  $H''$ .

There are several ways in which the causation of this declining rod contribution could be interpreted. It could be considered as an intrinsic property of the rod population of neural effects<sup>18</sup>. In this case it might be reasonable to expect that if the cone population could be in some manner obliterated or suppressed the declining rod curve could then be directly determined by experiment. The measurement of the flicker contour for a nocturnal animal (gecko) possessing only rods<sup>33</sup> definitely shows that retinal rods are not necessarily restricted to low intensity functioning. For human subjects the evidence from day-blind, color-blind subjects<sup>34</sup> is, of course, defective, as one does not know except by circular argument that the retina there possesses only rods—or that if it does these are the equivalents of the rods in a normal retina (in fact, the logic of the situation suggests that, if the argument concerned be accepted, they cannot be such equivalents).

<sup>32</sup> *Proc Nat Acad Sc*, 1936, 22, 412. *J Gen Physiol*, 1937-38, 21, 17, etc

<sup>33</sup> *Proc Nat Acad Sc*, 1938, 24, 538. *J Gen Physiol*, 1938-39, 22, in press

<sup>34</sup> Hecht, S, Shlaer, S, Smith, E L, Haig, C, and Peskin, J C, 1938, *Am J Physiol*, 123, 94

Another possibility is that, in the duplex flicker curve, the excitation of cone elements neurally inhibits the influence of rod units. This can be indirectly tested by examining the morphology of the declining rod curve as it is deduced for various animals in which the relative magnitudes, positions, and shapes of the rod and cone segments of the duplex flicker contour are diversely differentiated. The suggestive fact is brought out by such an examination that the more sharply the cone curve rises, the more abruptly does the rod contri-

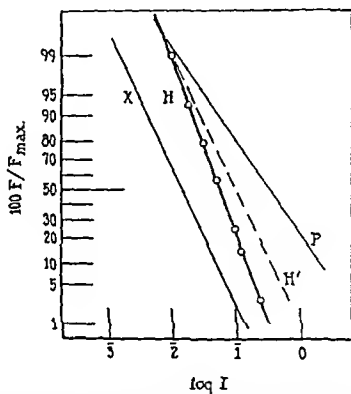


FIG 7 Curves taken to measure the dropping out of rod contributions with increase of  $F$  are obtained by the method of dissection illustrated in Fig 4. They adhere to probability integrals<sup>18</sup>. The points for  $H''$  are read from the curve in Fig 4. Discussed in the text.

bution decline<sup>24</sup>. If the location of the declining rod curve, most easily estimated by its  $\log I$  of inflection, moreover is more extensively removed from the rising branch of the rod curve the wider the separation of  $\tau'$  for rods from that for cones,<sup>24</sup> and its rate of decline is therewith found lessened. These relations are only slightly obscured by the presence of relatively enlarged rod populations.

The present data supply a rather significant test of this view, in



a fairly homogeneous group of cases. As Fig 7 shows,<sup>18</sup>  $\sigma'_{\log I}$  for  $X$  and for  $P$  are quite different. The declining rod curve for  $X$  falls off much more steeply, and at the same rate as for  $H'$ . The  $P$  cone curve rises more rapidly, but its rod population is smaller and more widely removed from the cone population. The  $H'$  rod curve is like the  $X$  in shape, and the  $H'$  rod and cone curves are shifted (as compared with  $X$ ) by about the same extent on the  $\log I$  axis. With  $H''$ , however, we find a rate of decline of the rod contribution which is much higher than for  $X$  and  $H'$ . In correlation with this, it is to be noticed that with  $H''$   $\tau'$  for cones is less widely removed from that for rods than is true with  $X$  or  $H'$ , or than in  $P$ , while the rod population is smaller than in  $X$ . The data are therefore consistent and suggest pretty plainly that if by some means the cone effects could be suppressed we would find no dropping out of the rod contribution (*cf* footnote 34), the pure rod curve of the gecko<sup>33</sup> shows no decline over the whole range of intensities open to test.

It is to be noted that these indications are entirely consonant with the conception that the quantitative properties of visual data, and specifically the data of flicker, are not determined by properties of the retina but measure on the contrary properties of central nervous events.

# V

## SUMMARY

The flicker response contour has been determined for several species and types of the teleosts *Xiphophorus* ( $X$ ) and *Platydocilius* ( $P$ ) under the same conditions. The curve ( $F$  vs  $\log I_r$ ) is the same for representatives of each generic type, but is different for the two genera. Its duplex nature is analyzable in each instance by application of the probability integral equation to the rod and cone constituent parts. The parameters of this function provide rational measures of invariant properties of the curves, which have specific values according to the genetic constitution of the animal. The  $F_1$  hybrids ( $H''$ ) of  $X$  *montezuma*  $\times$   $P$  *variatus* show dominance of the  $X$  properties with respect to cone  $F_{max}$  and  $\sigma'_{\log I}$ , but an intermediate value of the abscissa of inflection ( $\tau'$ ). The rod segment shows dominance of  $\sigma'_{\log I}$  from  $P$ , but an intermediate value

of  $F_{max}$  and of  $\tau'$  The composite flicker curve involves the operation of two distinct assemblages of excitable elements, differing quantitatively but not qualitatively in physicochemical organization, probably only secondarily related to the histological differentiation of rods and cones because almost certainly of central nervous locus, but following different rules in hereditary determination, and therefore necessarily different in physical organization The interpretation of the diverse behavior of the three parameters of the probability summation is discussed, particularly in relation to the physical significance of these parameters as revealed by their quantitative relations to temperature, retinal area, and light time fraction in the flash cycle, and to their interrelations in producing the decline of rod effects at higher intensities

It is stressed that in general the properties of the parameters of a chosen interpretive analytical function must be shown experimentally to possess the physical properties implied by the equation selected before the equation can be regarded as describing those invariant properties of the organic system concerned upon which alone can deduction of the nature of the system proceed The importance of genetic procedures in furthering demonstration that the biological performance considered in any particular case exhibits constitutionally invariant features provides a potentially powerful instrument in such rational analysis



# TEMPERATURE AND CRITICAL ILLUMINATION FOR REACTION TO FLICKERING LIGHT

## III SUNFISH

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### I

The curve (band) depicting the interdependence of flash illumination  $I$  and flash frequency  $F$  critical for response to visual flicker is shifted in the direction of lower intensities when the temperature of the animal is raised<sup>1</sup> Measurements with the insect *Anax* (larva)<sup>2</sup> and the turtle *Pseudemys*,<sup>3</sup> which exhibit the activity of only one homogeneous population of nervous elements concerned in determining the reaction to the threshold of flicker, show that the maximum value of  $F$  and the shape of the  $\log I - F$  curve are independent of temperature Only the position of the curve along the  $\log I$  axis is altered, this is also essentially true in the data from forms (arthropods) for which a structural factor, namely the convexity of the retinal surface,<sup>4</sup> mechanically introduces a distortion of the fundamentally simple character of the curve

The same invariance of shape and  $F_m$  when temperature is changed is apparent in data obtained with sunfish<sup>5</sup> and with *Tundulus*<sup>6</sup> These exhibit the typical vertebrate condition of a duplex curve for  $\log I$  vs  $F$  Here each of the two parts of the flicker response contour—commonly related to the functioning of retinal rods and cones,

<sup>1</sup> *J Gen Physiol*, 1936-37, 20, 393, 411 1938-39, 22, 311 *Proc Nat Acad Sc* 1938 24, 216, 1939, 25, 78

<sup>2</sup> *J Gen Physiol* 1936-37, 20, 393 1937-38, 21, 223

<sup>3</sup> *Proc Nat Acad Sc* 1938, 24, 125 *J Gen Physiol* 1938-39 22, 311

<sup>4</sup> *J Gen Physiol* 1937-38, 21, 223, 463, 1938-39, 22, 451

<sup>5</sup> *J Gen Physiol* 1936-37 20, 411

<sup>6</sup> *J Gen Physiol* 1938-39, in press *J Exp Zool*, 1939 in press

respectively—retains its value of  $F_{max}$  and of the shape constant independent of change in temperature. The *Fundulus* curve is important because of the large and prominent “rod” contribution.

The significance of these facts for the theory of the flicker response contour has been discussed previously.<sup>1</sup> The  $F - \log I$  curve is essentially a probability integral.<sup>7</sup> The elements of effect contributing to the summation of total effect proportional to  $F$  are elements of which the excitability fluctuates from moment to moment. At any instant the excitability of a given element is measured by the reciprocal of its exciting intensity. This view is consistent with the results of altering the area of a retinal surface<sup>8</sup> if it be correct that properties of a simple chemical system govern the slope of the curve for  $1/I$  as a function of temperature. This dependence is such that, for frequencies of heart beat, breathing movements, and the like, presumed to be dependent on and to measure the potential of a pacemaking process, the relative variation is independent of temperature when the apparent critical increment (temperature characteristic) is constant. Over the range of  $F$  which concerns us in the present experiments,  $\sigma I/I$  is constant, and so therefore is  $\sigma (1/I)/(1/I)$ , as will be pointed out, the ratio is independent of temperature.

The use of a particular analytical function, such as a probability integral, for the description of the flicker response contour could not be really justified by any mere processes of curve fitting, even if appropriate criteria could be found. The same restriction, of course, applies to the use of any other curve. The known properties of the measured variation of the critical intensity implicit in the data make classical criteria of curve fitting arbitrary.<sup>9</sup> Justification must be provided through and by means of tests which demonstrate that the parameters of the proposed function have the necessary physical properties, as well as that their number is necessary and sufficient. The probability integral, describing the data with adequate statistical fidelity, contains three “constants” (parameters) the ordinate of the upper asymptote ( $F_{max}$ ), the abscissa of its inflection point ( $\log$

<sup>7</sup> *Proc Nat Acad Sc*, 1937, 23, 71. *J Gen Physiol*, 1936–37, 20, 411, 1937–38, 21, 313, 1938–39, 22, 311. *Proc Nat Acad Sc*, 1938, 24, 125.

<sup>8</sup> *J Gen Physiol*, 1937–38, 21, 223.

<sup>9</sup> *J Gen Physiol*, 1937–38, 21, 313, 463.

$I_{inf}$ ), and the standard deviation of the summed frequency distribution ( $\sigma_{log I}$ ). Tests made by altering temperature, retinal area, and percentage light time in the flash cycle<sup>1 10</sup> show clearly that these parameters can be separately controlled, and that (so far as present evidence extends)<sup>11</sup> these particular three parameters are sufficient as well as necessary. A rigorous test of the biological reality of a physical basis for these parameters is available by way of genetic experiments, cross breeding experiments show<sup>1</sup> that they can behave as specific genetic entities.

To learn something about the mechanism governing the excitability we may investigate quantitatively the way in which the abscissa of the inflection ( $\log I_{inf}$ ) point of the curve depends upon the temperature. This parameter is the abscissa of the mode (median) of the frequency distribution of the  $\log I$  thresholds of the population of sensory effects. Since the form of the  $F - \log I$  curve does not change with alteration of temperature, nor the upper asymptote, the same kind of information can be obtained from the behavior of the critical illumination at any fixed level of  $F$ . With elevation of temperature the frequency distribution,  $i.e. dI/d \log I$  as a function of  $\log I$ , is merely moved to a lower position on the  $\log I$  axis. The extent of this shift, for a given change of temperature, is the same for both sections of the curve, since their shape constants do not change.

The origin of the log form of the function may be explained in several ways. We may take it for granted that at any instant the frequency distribution of mean individual excitabilities ( $1/I_1$ ) would be normal, since obviously a large number of elements is involved. Reaction, at a fixed (low)  $F$  would then require the production of a certain summed quantity of excitation (proportional to  $F$ ). But the excitation process occupies a finite time interval, and within this period the individual excitabilities fluctuate at random. The assumption to be made is that for very low intensities, effect ( $= kF$ ) and  $I$  will be directly proportional, a small increase of effect to be

<sup>10</sup> *J Gen Physiol* 1937 38 21, 313, 463

<sup>11</sup> *J Gen Physiol*, 1935-36 20, 503 *Proc Nat Acad Sc*, 1936 22, 412

<sup>1</sup> *J Gen Physiol*, 1936-37, 21, 17 1938-39, 22, 463 *Proc Nat Acad Sc* 1938, 24, 221

obtained will be directly proportional to the increase of intensity, but inversely proportional to the intensity level

$$dE = k_1 dF = k_2 \frac{dI}{I}$$

so that we will have to deal, not with a frequency distribution of  $dI$ , but of  $dI/I$  and thus of  $d \log I$ . This is required by the fact of the "saturation" of the  $F - I$  curve, and holds equally well if  $dE$  is taken as proportional to the excitability  $d(1/I)$ , since  $-\frac{dI}{I^2} - \frac{1}{I} = -\frac{dI}{I}$  (*i.e.*, the direction of the scale is merely reversed)

In another way, if under a given intensity  $I$  the occurrence of effect-contributions from an element follows a Poisson distribution, for which there is experimental evidence, the log frequency distribution is derived directly

Essentially the same argument applies to other dynamically similar situations, as in strength-duration data for excitation of nerve<sup>13</sup>. In that case lowering the temperature also raises the abscissa ( $\log t$ ) of the inflection point of the curve ( $1/C$  vs  $\log t$ ) but in addition raises the maximum (*i.e.*, decreases the rheobase) because a larger number of elements of excitation is made available in the longer critical time, the shape of the curve, measured by  $\sigma'_{\log t}$  with  $1/C$  on a percentage scale, is unchanged

## II

The quantitative dependence of the shift of the  $F - \log I$  curve on change of temperature should provide evidence as to the nature of the control of excitability. For *Pseudemys* the relationship is of a kind frequently encountered in biological systems,<sup>3</sup>  $1/I_m$  for a fixed  $F$  follows the Arrhenius equation, with  $\mu = 27,000$  calories below  $30^\circ$  and  $\mu = 12,000$  above  $30^\circ$ . These values of the temperature characteristic and of the critical temperature are often found. With *Enneacanthus* (sunfish), however,<sup>2</sup> observations at three temperatures ( $12.4^\circ$ ,  $21.5^\circ$ ,  $27.3^\circ$ ) gave on an Arrhenius grid ( $\log 1/I_m$  vs  $1/T_{obs}$ ) a plot convex upward instead of rectilinear. Such instances are unusual, and led us to conclude<sup>2</sup> that the flicker response phenomenon

<sup>13</sup> Cf *Proc Nat Acad Sc*, 1937, 23, 71 *J Gen Physiol*, 1937-38, 21, 223

is too complex to provide significant data for description in this way. This could not be entirely due to the presence of both rod and cone contributions to the flicker curve, since somewhat similar complications appeared in the case of the dragonfly *Anax* (nymphs). The behavior of the flicker response contour for the sunfish was therefore reinvestigated, since with the occurrence of an intermediate critical temperature<sup>14</sup> observations at a number of closely spaced points are necessary to decide if a change of temperature characteristic is involved. Instances in which the apparent critical increment is greater over the range above a critical temperature are comparatively rare, but are not unknown.<sup>16</sup> Such a situation would account for certain features of the original data on the sunfish.<sup>1</sup> It should also be possible to determine if the shift of the rod portion is really of the same kind as for the cone part of the curve. This point has a certain theoretical importance.<sup>16</sup> It turns out that the detailed study of the reactions of the sunfish does make it clearer why the initial observations were incompetent to decide this matter.

The experimental conditions were the same as those discussed in some detail in our earlier papers.<sup>1</sup> So also were the method of obtaining the measurements and the procedure for calculation of the results. In the present lot of *Emneacanthus*, as in several earlier series<sup>17</sup> there is no evidence of persisting individualities among the several fishes used throughout the sets of measurements. The relative rank orders of reactivity are randomly distributed, and show no correlations in successive sets of readings. The methods of work are sensitive enough to reveal rather delicate differences of this kind, in lots of other organisms when they do occur.<sup>18</sup> This provides an essentially objective control on the methods of observation. The present data are demonstrably homogeneous by this test. The relation of the scatter of the critical intensities to the mean intensity is precisely that found in the earlier series.  $PE_{1/2}$  is directly proportional to  $I_m$ , the proportionality constant is not demonstrably different from that found in the earlier series (Fig. 1). The different temperatures were used in a completely random order. Observations on any one day were at  $F = 4$  and  $F = 25$  at the same

<sup>14</sup> Cf. *J. Gen. Physiol.*, 1924-25, 7, 123, 189, 1925-26, 8, 525, etc.

<sup>15</sup> *J. Gen. Physiol.*, 1930-31, 14, 421.

<sup>16</sup> *J. Gen. Physiol.*, 1928-29, in press.

<sup>17</sup> *J. Gen. Physiol.* 1935-36, 19, 495, 1936-37, 20, 211, 411, 1937-38, 21, 313  
*Proc. Nat. Acad. Sc.*, 1938, 24, 221.

<sup>18</sup> *J. Gen. Physiol.*, 1936-37, 20, 363, 393, 1938-39, in press.



temperature Repetition of readings at a particular temperature, after some days' use at other temperatures, shows unexceptionably close agreement There is a distinct suggestion that on any one day the two sets of readings (at  $F = 4$ ,  $F = 25$ ) may tend to depart slightly in the same direction, so that both may be "too high" or "too low" by comparison with the others This could perhaps be due in part to slight errors in temperature adjustment, but there is probably involved as well a real day-to-day fluctuation in the whole lot of fishes Values obtained several years ago, with of course a different lot of individuals, uniformly show a slightly higher value of  $I_m$  at  $F = 4$  The difference is not really signifi-

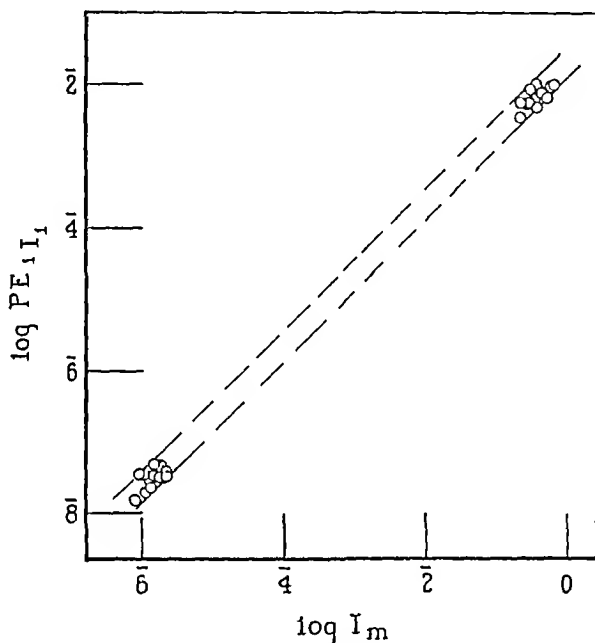


FIG 1  $P E_{I_1}$  is directly proportional to  $I_m$  (*i e*, the slope of the log *vs* log plot = 1), independently of the temperature Data in Table I (new determinations only)

cant, however The variability of  $I_1$ , the mean critical intensity for reaction, tends to be a little higher for our earliest series<sup>1</sup> These data are given for comparison in Table I

The two most extensive departures (at  $F = 4$ ,  $21.5^\circ$  and at  $F = 25$ ,  $23.9^\circ$ , see Table I and Figs 2 and 3) occur at points for which an extremely small difference in the adjustment of the diaphragm controlling the intensity of illumination (*cf* footnote 1) has a large influence upon the recorded intensity, owing to the slope of the calibration curve, a difference of  $0.3 \text{ mm}$  in the diaphragm opening would obliterate the departures, and this is actually less than the difference between mean values in duplicate sets of  $3 \times 10$  readings These departures are therefore not significant

## III

Data were obtained at two flash frequencies,  $F = 4$  per sec and  $F = 25$  per sec, with a light time proportion of 50 per cent ( $t_c$ ,  $t_L = t_D$ ), at  $t^\circ = 21.5$ . These two flash frequencies were chosen so as to fall within the exclusively rod and the exclusively cone parts of the

TABLE I

Mean critical flash intensities for response to flicker, at two flash frequencies ( $F = 4$ ,  $F = 25$ ), as a function of temperature, for the sunfish *Enneacanthus*. \*taken from earlier measurements<sup>17</sup> 30 measurements at each point (3 on each of the same 10 individuals).  $I$  in millilamberts,  $PE_{1/1}$  is the  $PE$  of the dispersion, computed as described in the text.  $F$  is in flashes per sec, the flash cycle has equal light time and dark time ( $t_L = t_D$ ). See Figs 2 and 3

$t^\circ C$	$F_4$		$F_{25}$	
	$\log I_m$	$\log PE_{1/1}$	$\log I_m$	$\log PE_{1/1}$
12.2	$\bar{6} \ 3423$	$\bar{8} \ 5833$	$\bar{1} \ 7613$	$\bar{3} \ 9793$
	$\bar{6} \ 3424$	$\bar{8} \ 5034$	$\bar{1} \ 7691$	$\bar{2} \ 0402$
12.4	$\bar{6} \ 3392^*$	$\bar{7} \ 0253$	$\bar{1} \ 7755^*$	$\bar{2} \ 4281$
13.6	$\bar{8} \ 3004$	$\bar{8} \ 5974$	$\bar{1} \ 7263$	$\bar{3} \ 9665$
15.0	$\bar{6} \ 2615$	$\bar{8} \ 5274$	$\bar{1} \ 6815$	$\bar{3} \ 8963$
16.4	$\bar{8} \ 2317$	$\bar{8} \ 6712$	$\bar{1} \ 6585$	$\bar{3} \ 8074$
18.0	$\bar{8} \ 2169$	$\bar{8} \ 5181$	$\bar{1} \ 8225$	$\bar{3} \ 8150$
19.6	$\bar{8} \ 1821$	$\bar{8} \ 7126$	$\bar{1} \ 5913$	$\bar{3} \ 7388$
	$\bar{6} \ 1807$	$\bar{8} \ 6057$		
21.5	$\bar{6} \ 1784^*$	$\bar{8} \ 7160$	$\bar{1} \ 5631^*$	$\bar{2} \ 7993$
	$\bar{6} \ 1735^*$	$\bar{8} \ 6610$	$\bar{1} \ 5566^*$	$\bar{3} \ 8779$
	$\bar{6} \ 1648$	$\bar{8} \ 4789$	$\bar{1} \ 5585$	$\bar{3} \ 6684$
22.7	$\bar{8} \ 1266$	$\bar{8} \ 3753$	$\bar{1} \ 5050$	$\bar{3} \ 9116$
23.9	$\bar{6} \ 0781$	$\bar{8} \ 2962$	$\bar{1} \ 4404$	$\bar{3} \ 9113$
25.5	$\bar{8} \ 0488$	$\bar{8} \ 5690$	$\bar{1} \ 4079$	$\bar{3} \ 7317$
27.2	$\bar{7} \ 9634$	$\bar{8} \ 2853$	$\bar{1} \ 3819$	$\bar{3} \ 7567$
27.3	$\bar{7} \ 9708^*$	$\bar{8} \ 5705$	$\bar{1} \ 3609^*$	$\bar{2} \ 2201$
			$\bar{1} \ 3602^*$	$\bar{3} \ 8543$
29.0	$\bar{7} \ 9064$	$\bar{8} \ 1923$	$\bar{1} \ 2882$	$\bar{3} \ 5388$

duplex flicker response contour for *Enneacanthus*<sup>1</sup>. The results are given in Fig 2, and in Table I. The evidence for absence of change of  $F_{max}$  and of shape of curve when the temperature is altered is fairly conclusive<sup>14</sup>. We may therefore accept the change of  $I_m$  at fixed  $F$  as a legitimate index of the shift of the whole function.

Fig 2 makes it clear that the behavior of the excitability ( $1/I_m$ ) as a function of temperature is somewhat unusual. There is a critical temperature near  $20^\circ\text{C}$ , and above this the temperature characteristic is *greater* than at lower temperatures. There is also a distinct, significant difference in the data for  $F = 4$  and  $F = 25$ . The important

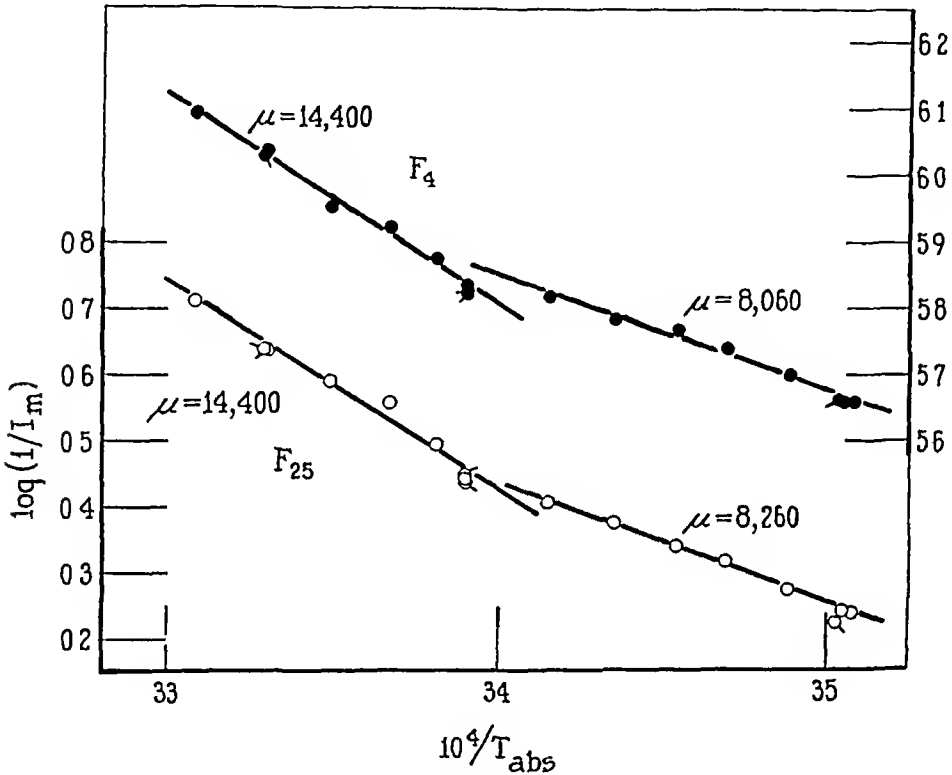


FIG 2 The rectilinear dependence of  $\log 1/I_m$  (at fixed  $F$ ) on  $1/T_{abs}$  gives essentially the same slopes for  $F_4$  and  $F_{25}$ , observations from older series of observations are indicated by tags (see text). The dislocation of the two halves of the plot for  $F_4$  is discussed in the text. Data in Table I. The departures are not significant (see text).

indication is that the temperature characteristics for  $I_m$  at the two flash frequencies are the same, but that for  $F = 4$  there occurs a shift of the curve above the critical temperature (*ca*  $20^\circ\text{C}$ ) which is not involved for the measurements at  $F = 25$ . Fig 3 brings this out in a simple way. If  $\mu$  is the same for the two series of readings at  $F = 4$  and  $F = 25$ , above  $20^\circ$ , and below, then multiplication of one

of these sets by a constant should, of course, bring the two into coincidence—provided the rod and cone curves suffer the same kind of change on passing beyond the critical temperature Fig 3 shows

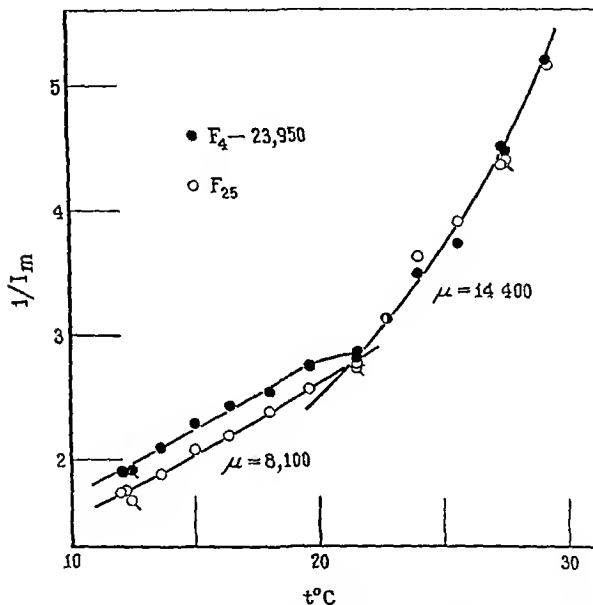


FIG 3 The values of  $1/I_m$  plotted in Fig 2 are brought together for comparison by dividing those at  $F_4$  by a constant ( $\approx 23,950$ ), which shows the identity of the curves for the upper range of temperatures ( $>20^\circ\text{C}$ ) and the greater dislocation of the  $F_4$  measurements at  $ca\ 20^\circ$ . The curves are the same as those shown in rectilinear form in Fig 2

that the situation is more involved. Above  $t^\circ \approx 20$ ,  $\mu \approx ca\ 14,400$ , below this temperature  $\mu \approx ca\ 8,300$ , this obtains for both  $F = 4$  and  $F = 25$ , but for  $F = 4$  above  $t = 20^\circ$  the whole curve has been

moved as a unit toward lower values of  $1/I_m$ . When the values of  $1/I_m$  for  $F = 4$  have been brought into coincidence with those for  $F = 25$ , above  $t^\circ = 20$ , by division of all the values with an appropriate constant, the curve for  $F = 4$ , below  $t^\circ = 20$ , lies to the left (Fig 3)

The recognition of critical temperatures<sup>14,15</sup> has been upon the basis of (1) abrupt changes of  $\mu$  on either side of certain temperatures, (2) abrupt shifts of frequency or rate of biological performance without change of  $\mu$ , at the same temperatures, and (3) the occurrence of combinations of (2) and (3). The argument has been outlined in a recent paper<sup>19</sup> and need not be recapitulated. The reality of the evidence in the present data is patent. It is of interest that the shift suffered by the rod curve at  $20^\circ$  reinforces in an unequivocal way the interpretation of the Arrhenius graph as composed of two intersecting straight lines, rather than as a continuous curve with upward concavity. It would be difficult to adjust such a curve to the data for  $F = 25$ , but cases of this kind are known<sup>20</sup>. They signify the concurrent participation of parallel, additive processes having unlike temperature characteristics, and are of course by no means inaccessible to interpretation<sup>21</sup>. The nature of the present data for  $F = 4$  (Figs 2 and 3) makes it unnecessary to discuss the question here, since the "break" identified at *ca*  $20^\circ$  is obviously real. It is curious that a previously known instance of this kind<sup>15</sup> also involves the rather infrequent  $\mu = 14,400$ .

#### IV

We are concerned with the significance of this evidence in two particular respects

1 It is clear that the calculation of a temperature coefficient ( $Q_{10}$ ) for two arbitrary temperatures, say  $t = 17^\circ$  and  $t = 20^\circ$ , could show an apparently significant difference for the rod and cone portions

<sup>19</sup> *J Gen Physiol*, 1938-39, in press

<sup>20</sup> An example is found in Korr's data (*J Cell and Comp Physiol*, 1937, 10, 461) on the respiration of fertilized sea urchin eggs. An analysis has been given by one of us in another place

<sup>21</sup> Cf, e g, Norrish, R. G. W., and Rideal, E. K., 1923, *J Chem Soc*, 123, 696, 1689, 3202

of the flicker curve. It is equally clear that the difference would be without meaning or use, and that if it were to be employed as an argument for the occurrence of distinct chemical organization of events supposed to underlie the determination of the two parts of the duplex curve of visual functioning, the result would be worse than misleading. Such an argument would be simply wrong, because it is evident that the apparent critical increments for  $1/I_m$  in the rod and cone parts of the curve are the same. Thus there is no real basis for assuming a qualitative difference in chemical organization to be reflected in the events determining the two parts of the duplex flicker contour. A difference of this sort is implied in a treatment of the data on the sunfish<sup>1</sup> offered by Hecht,<sup>2</sup> we had already pointed out<sup>1</sup> that this alternative interpretation seems to be without cogency or validity.

2 Although there is from the temperature experiments no evidence for difference in the chemical organization of the neural elements responsible for the rod and cone portions of the curve for the sunfish, there is adequate evidence of the duplexity of the visual function. The shift of the rod curve on either side of  $20^\circ$  is demonstrably different from that for the cone part. The flicker curve has been found to be essentially a *band*, defining the limits of  $F$  and  $\log I$  within which (under given conditions) there obtains a certain probability (defined by measured  $\sigma_p$  and  $\sigma_I$ ) that response will be observed. The values of  $I_m$ , for a fixed  $F$  and  $t_L/t_D$ , with temperature as variant, measure the "driving force" required to eventuate the index response,<sup>1,3</sup> and  $1/I_m$  is thus a valid measure of excitability. The elements activated by the driving force of repeated flash intensities, the summated effects of which are responsible for response, are presumed to be central nervous in location. That the two groups of these elements involved in determining the probability of occurrence of the index reaction should be assumed to be in some manner connected with the duplexity of retinal structure apparent in typical vertebrates there is abundant evidence.<sup>23</sup> That this necessarily supports the view that the quantitative properties of visual data, as for example the

<sup>23</sup> Hecht S, *J Applied Physics*, 1938, 9, 156

<sup>22</sup> Cf Hecht, S *Physiol Rev* 1937, 17, 239 *Proc Nat Acad Sc*, 1938, 24, 125

data of response to flicker, directly image the properties of retinal rods and cones, there is no evidence whatever. The facts show that in vertebrates such as *Emneacanthus* there are involved two groups of neural elements which are implicated in recognition of visual flicker. The results of breeding experiments<sup>12</sup> show that the parameters of the functional expression of these groups of elements can behave in a simple, independent manner in inheritance. A separate physical substratum is therefore to be postulated for each. The parameter basically concerned in the present experiments is the abscissa of the inflection of the  $F - \log I$  curve. Changes in this quantity with change of temperature measure changes in the mode (median) of the frequency distribution of  $\log I$  thresholds for excitation of elements, under conditions otherwise constant. There is no reason for surprise at finding that the two groups of elements, whether or not to be specifically identified with the resultants of rod and cone excitation respectively, may be somewhat differently affected by passage through and beyond a critical temperature, since (as the *genetic* results show) a physically different substratum must be assumed for each of them. But so far as can be indicated by the evidence of their excitability temperature characteristics ( $\mu$ ) the physicochemical properties of the elements of excitation in the two groups are the same. This is entirely consistent with the somewhat surprising fact that for purely cone (turtle) and rod (gecko)<sup>24</sup> retinas the  $F - \log I$  contours have almost indistinguishable values of  $\log I_{inf}$ , of  $\sigma'_{\log I}$ , and of intensity range.

We are indebted to Dr Gertrud Zerrahn-Wolf for assistance in the observations and experiments.

#### SUMMARY

For the sunfish *Emneacanthus* the mean value of the critical illumination for response to visual flicker at constant flash frequency (with light time = dark time) is related to temperature by the Arrhenius equation. The temperature characteristic for  $1/I_m$  is different above and below 20°C. In each range (12° to 20°, 20° to 30°) the temperature characteristic is the same for rod and cone segments.

<sup>24</sup> Data in course of publication

of the duplex flicker response contour 8,200 and 14,400. This makes it difficult, if not impossible, to consider that the two groups of elements are organized in a significantly different way chemically. For the presumptively rod connected elements implicated in response to flicker, the curve is markedly discontinuous, so that the high and low temperature parts are dislocated, whereas for the cones they are not. This is entirely consistent with other (*e g*, genetic) evidence pointing to their separate physical substrata.

The uncommon exhibition of a higher  $\mu$  over a higher range of temperature, previously found, however, in a few cases, together with the different relations of rod and cone effects to the critical temperature, explain aspects of these data which in earlier incomplete measurements<sup>1</sup> were found to be puzzling.





## THE KINETICS OF PENETRATION

### XVI THE ACCUMULATION OF AMMONIA IN LIGHT AND DARKNESS

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(Accepted for publication, December 30, 1938)

In a former paper<sup>1</sup> the accumulation of ammonia<sup>2</sup> by *Valonia macrophysa*, Kutz, was discussed. In the present paper we compare the rates, and steady states, in cells exposed to sea water containing ammonia in light<sup>3</sup> (regular succession of daylight and darkness) and in the dark. At the same time the pH changes have been examined by an improved method which avoids the loss of gas from the sap.

The experiments were carried out in Bermuda in the winter of 1937-38 at the Bermuda Biological Station for Research.

#### EXPERIMENTAL

In these experiments the cells were exposed in large jars to the sea water containing ammonia. In the dark experiments the glass jars were made dark by layers of adhesive tape covered with a thick layer of either black paint or a very opaque aluminum paint. In some cases however stoneware jars were used. In all cases the jars were stoppered and were immersed up to their necks in flowing salt water from the Biological Station salt water system. The temperature remained fairly constant at  $17^{\circ}\text{C} \pm 1^{\circ}\text{C}$  during the course of the experiments.

Analyses for potassium and sodium were each carried out on 0.1 ml. samples as described in previous papers<sup>1</sup> except that in place of the Kublmann micro balance, a Becker semi micro balance was used. Weighings were made to 0.02 mg. which in most cases represented a weighing error of less than 0.5 per cent. This accuracy was quite sufficient in view of the variability of the material.

The ammonia analyses were carried out by means of Nessler's reagent using the method described in the previous paper. The quantity of sample varied from

<sup>1</sup> Jacques A G and Osterbout W J V *J Gen Physiol*, 1930-31, 14, 301

<sup>2</sup> To prevent misunderstanding we shall define ammonia as  $\text{NH}_3 + \text{NH}_4^+ + \text{NH}_4\text{OH}$  i.e., the total amount found by analysis. We shall speak of  $\text{NH}_3$  as undissociated ammonia.

2 ml to 0.1 ml, care being taken to operate always on the linear portion of the extinction coefficient-concentration calibration curve. This required in many cases dilution of the sample to 104 ml in place of the usual 52 ml.

The volume was determined on selected groups of a few cells believed to be representative of all the cells used in an experiment.

The pH of the sap was determined by a new method. It has been noticed repeatedly in the past that the pH of the sap rises immediately after extraction. This seems to be connected in part with the loss of carbon dioxide, and the process is hastened by anything which furnishes nuclei on which the gas bubbles can form. Metal surfaces fall in this class and mere passage of the sap through a stainless steel hypodermic needle, during extraction, is sufficient to change the pH appreciably. In the new method the loss of gas was avoided. Tuberculin syringes of pyrex glass, usually of 0.25 ml capacity, were used. These were fitted with all-glass needles, and each syringe had a small pyrex glass bead in it to mix the contents after the sap and indicator had been drawn in.

The procedure was as follows. With piston pushed in a cell was impaled and its sap was forced into the syringe by squeezing the cell. This carried the small amount of air in the needle into the syringe and also served to wet the barrel and piston and to move the piston out. The piston was then pushed in, expelling the air and filling the needle with sap, which might, however, have lost some of its gas during its brief exposure to the air. Another charge of sap was then forced into the syringe pushing the piston but no air ahead of it. This and one more charge were used to wash out the syringe and the charge of sap to be tested was then forced in. The required amount of indicator was then drawn into the needle and was forced into the barrel by impaling another cell and squeezing the sap into the needle. The charge of sap and indicator, still free of air bubbles, was then mixed by means of the glass bead. By this procedure we obtained a sample of sap mixed with indicator, which had not been exposed either to air or to a metal needle after extraction.

The pH could be determined by comparing the test syringe with buffers and indicators in similar syringes, but we wished to keep on using the Hellige double-wedge colorimeter which has been so satisfactory. Accordingly a special carrier was devised to hold the syringe in the colorimeter in approximately the position usually occupied by the conventional glass cell. A simple plano-convex lens was placed between the syringe and the eyepiece. This had the effect of spreading the emergent beam from the syringe so that only a narrow band passing through the middle of the syringe was viewed. Since the syringe is cylindrical the intensity of the color of the band necessarily decreases from the center. But by selecting only a narrow band the decrease in color to the edges of the field of view was made inappreciable.

The ordinary tuberculin syringe has two other disadvantages. The figures etched on the barrel must be kept out of the field by turning the syringe until they are out of way, and the inner surface of the barrel is ground so that even when it is filled with liquid some scattering occurs. This has the effect of slightly

reducing the brilliance of the color, but one soon learns to discount this slight effect. Doubtless the difficulty could be overcome by using syringes made of Jena KPG tubing which is so accurately fabricated that barrel and piston do not have to be ground to a fit. The pH's were read off a calibration curve constructed by means of buffers treated in the syringe in the same way as the unknown sample.

## RESULTS

Four experiments comparing the rates of accumulation in normal light<sup>3</sup> and darkness are discussed.

*Experiment 1* — Cells were exposed to sea water containing 0.00175 M ammonia at the normal pH of sea water.<sup>4</sup> The results of this experiment are given in Table I and Fig. 1. The behavior of sodium and potassium will be considered in detail. In this experiment the pH was determined parallel with the ammonia changes by the method described in this paper. The results of the pH measurements together with the rate of increase of ammonia have been plotted in Fig. 3. Since the method is rather wasteful of cells the pH was not determined in Experiments 2, 3, and 4.

*Experiment 2* — Cells were exposed in light and dark to sea water containing 0.0025 M ammonia at normal pH. The results are given in Fig. 1.

*Experiment 3* — Cells were exposed in light and dark to sea water containing 0.0025 M ammonia at normal pH. The dark group was subjected in this experiment to extremely dark conditions. The results are given in Fig. 2.

*Experiment 4* — As in Experiment 3, except that before exposure to sea water containing ammonia the cells of both groups were subjected to a preliminary period of darkness in ordinary sea water. (See Fig. 2.)

Before considering the rates of entrance let us consider the pH measurements. From Fig. 3 it appears that soon after the cells were exposed in normal light to the sea water containing ammonia the pH rose by a rather small but quite definite amount (by 0.4 pH), and that the new pH level at pH 6.10 was maintained until the end of the experiment. In darkness also the pH rose promptly though not quite

<sup>3</sup> Normal light means the regular daily succession of light and darkness.

<sup>4</sup> The pH of the body of the sea water was 8.2 but it varied in the immediate neighborhood of the cells depending on illumination, etc.

so much as in the light. But in this case a slow decrease then followed and at the end of the experiment the pH of the sap was about that of normal sap, in spite of the fact that the sap now had 0.05 M

TABLE I  
*Accumulation of Ammonia in Sea Water Containing 0.00175 M Ammonia  
(Experiment 1)*

Days	Volume	Concentration			$P'_L$	$P'''_L$
		Ammonia	Potassium	Sodium		
Light group						
	cc	M	M	M		
0	1 98	0 0006	0 4846	0 1475	—	
2 5	2 01	0 0274	0 4770	0 1520	0 0039	0 0027
5	2 02	0 0493	0 4420	0 1576	0 0035	0 0026
7	2 04	0 0600	0 4274	0 1600	0 0031	0 0023
10	2 06	0 0850	0 4013	0 1649	0 0031	0 0026
13	2 09	0 0978	0 3780	0 1786	0 0027	0 0024
15	2 10	0 1178	0 3725	0 1692	0 0029	0 0028
20	2 13	0 1339	0 3400	0 1779	—	
26	2 16	0 1319	0 3143	0 1832		
Dark group						
	Adjusted volume*				$P_D$	$P'''_D$
0	1 98	0 0006	0 4846	0 1475	—	—
2 5	1 97	0 0184	0 4766	0 1425	0 0097	0 0076
5	2 00	0 0253	0 4628	0 1524	0 0067	0 0058
7	1 94	0 0286	0 4729	0 1429	0 0077	0 0050
10	1 97	0 0395	0 4760	0 1360	0 0054	0 0062
13	2 01	0 0464	0 4515	0 1516	0 0049	0 0077
15	1 98	0 0477	0 4509	0 1539	0 0043	0 0073
20	2 02	0 0494	0 4361	0 1572	0 0035	0 0067
26	2 02	0 0518	0 4366	0 1415	—	—

\* Adjusted by multiplying the measured volume in the dark group by 1.98 ÷ 1.80 so as to make it possible to start light and dark group "moles" curves at the same zero point

$\text{NH}_4^+$  and had lost a corresponding concentration of potassium. It appears then that there is little correlation between the concentration of ammonia in the sap and the pH. This is particularly clear in the

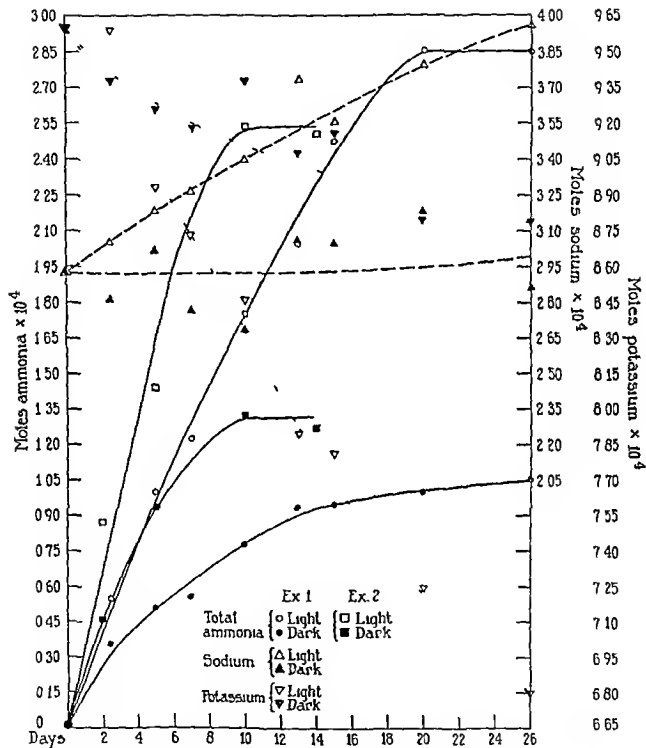


FIG 1 Accumulation of ammonia in light and darkness Experiments 1 and 2  
 The curves are drawn free hand to give an approximate fit

dark group where after the first rapid pH rise the ammonia concentration and the pH moved in general in opposite directions

These results contradict effectively a suggestion made in a former paper that the presence of a relatively high concentration of ammonia in the sap results in the buffering of the sap by ammonium salts at a somewhat higher level than that of normal sap. It now seems much more likely that the change in pH may be associated with relatively small amounts of basic ammonia in the sap

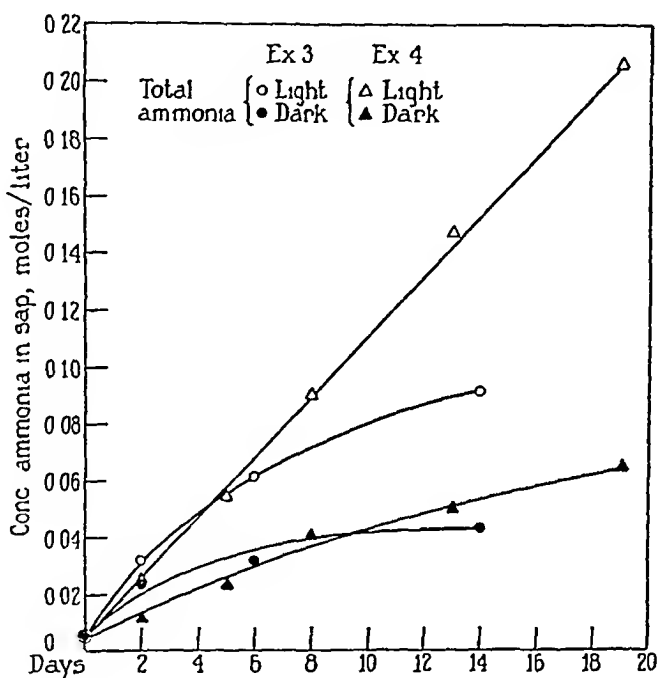
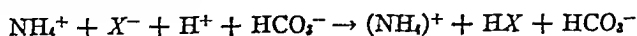


FIG 2 Accumulation of ammonia in light and extreme darkness. Experiments 3 and 4. The curves are drawn free-hand to give an approximate fit.

In the accumulation of ammonia, tending to raise the pH of the sap, we suppose that we have the entrance of ammonia as  $\text{NH}_4\text{X}$ ,<sup>5</sup> a form equivalent to entrance as a base since each molecule of  $\text{NH}_4\text{X}$  is decomposed at the sap-protoplasm interface by an acid stronger than  $\text{HX}$  (probably  $\text{CO}_2$ ) thus,



<sup>5</sup> Osterhout (Osterhout, W J V, *Proc Nat Acad Sc*, 1935, 21, 125) has suggested that ammonia entrance is preceded by a reaction between ammonia in the sea water and an acid  $\text{HX}$  in the protoplasm

Since  $HX$  is by assumption a very weak acid nearly insoluble in water it does not figure in the pH of the sap. And tending to lower the pH we have (a) the loss of potassium as  $K\lambda$  which is equivalent to the loss of a base, since  $KOH$  from the sap unites with  $H\lambda$  in the protoplasm to form the  $KX$ . (b) The elaboration in the protoplasm of water soluble acids which pass into the sap. Carbon dioxide is the most conspicuous of these but others probably play a part. This process which goes on constantly is in part offset by the outward diffusion of  $CO_2$  from the sap to the sea water. In the light photo

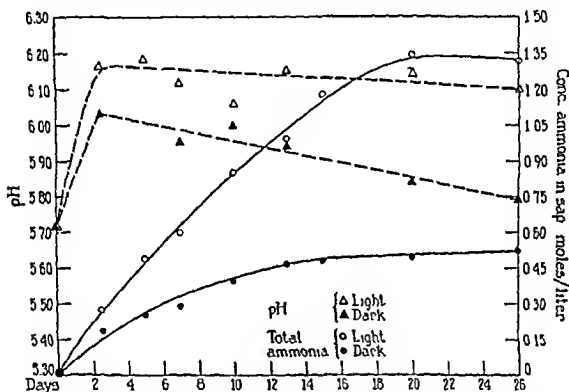


FIG 3 Change of pH of sap during accumulation in light and in darkness. The curves are drawn free hand to give an approximate fit.

synthesis, which we assume to be confined chiefly to the sea water protoplasm interface, removes the  $CO_2$  there and as a result the gradient of concentration of  $CO_2$  from the sap to the sea water is increased. This results in a more rapid loss of  $CO_2$ .

The pH of the sap is thus the resultant of several processes. The mixture of buffer systems is probably quite complex. But we may say confidently that the increase of free base will raise the pH and the decrease of base will decrease it. Such a change might possibly be brought about by the replacement of  $KOH$  by an equivalent amount



of  $\text{NH}_3$  with the system moving towards a higher pH due to the introduction of a new buffer system. Instead we believe that the results are more readily explained by assuming that the pH rise in both the light and dark groups depends on the rapid entrance of ammonia in a form equivalent to the base, without the loss of a corresponding amount of potassium as base. In the accumulation of ammonia we notice that in general at the very start of the process the total concentration of cations in the sap increases a little. We associate this in part with the gain of some ammonia without an equivalent loss of potassium.

In both groups the same process operates to raise the pH at the start, but in the dark group because the  $\text{CO}_2$  diffuses out of the sap more slowly than in the light the effect of the entrance of the excess of ammonia over the potassium lost, tends to be nullified. Hence the slow decrease of pH.

We must now consider to what extent the pH measurements are trustworthy. We have to face the fact that the sap is poorly buffered. For example, probably the buffer system of normal sap is due to sodium bicarbonate and potassium bicarbonate. But the total carbon dioxide concentration of the sap, according to Osterhout and Dorcas,<sup>6</sup> is only 0.0002 M of which, at pH 5.72,<sup>7</sup> one third is salt and the rest free  $\text{CO}_2$ . The final concentration of indicator used was 0.0001 M, or about half that of the buffer system. It would not be surprising therefore if the buffer equilibrium should be seriously upset by the indicator. A practical test of this possibility was made in New York by determining without gas loss the pH of a sample of sap with two concentrations of indicator. The same value was obtained in both cases. But this might mean that the buffer capacity of the indicator is too great to be upset by the addition of sap. This could hardly have been so in the present case since there was an obvious change in the color of the indicator as it was added to the sap. On the whole therefore we believe that the measurements give a true picture of the trend of the pH changes, and roughly of their magnitude.

<sup>6</sup> Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, 9, 255.

<sup>7</sup> This value was obtained in the present paper. The proportion of salt to base was calculated by the Henderson-Hasselbalch equation, using for  $\text{pK}'_1$  the value of 6.02 according to the latest results of MacInnes and Shedlovsky.

Considering now the rate experiments, all four lead to the conclusion that the rate of entrance of ammonia is strongly influenced by light. Thus the rate of entrance of moles (concentration  $\times$  volume) was about 2.5 times as fast in light as in darkness. This was hardly a question of the greater rate of growth of the cells in light. On a concentration basis in which we ignore the difference in growth rate the rate of concentration increase was about twice as fast in two experiments, and about three times as fast in the other two.

From Experiment 3 we can draw the additional conclusion that accumulation can go on even in total darkness. In this experiment in order to rule out the possibility that any daylight at all might get to the dark group the hottles in which the cells were exposed were covered with thick layers of adhesive tape painted black. These bottles were placed in sea water in a ten gallon stoneware crock with a cover. This was kept in a room sufficiently dark to be used as a photographic dark room. To keep the cells at about the same temperature as the light group, the stoneware crock was immersed in a bath through which a flow of salt water was maintained. In spite of the absence of light the cells accumulated ammonia and the rate was comparable with that observed in Experiment 2 in which the darkness was not so complete. The rate in darkness was about half that in normal light.

The cells of Experiment 3 were transferred directly from a large collection which was kept under normal light. It seemed not impossible therefore that they might have stored energy during this period which would be available for accumulation for a considerable period after the withdrawal of the light. In order to minimize this factor, if it should exist, the cells of Experiment 4 were first subjected to a preliminary exposure to normal sea water in total darkness for 14 days. This treatment had no visible effect on the rate of accumulation in the dark. In the light accumulation took place somewhat faster than in Experiment 3. However, Experiments 3 and 4 are not directly comparable and we cannot say definitely that the preliminary dark treatment rendered the cells capable of accumulating ammonia faster in the light.

All the curves for the accumulation of ammonia in Fig. 1 flatten out towards the end of the exposure. In the light where the cells continue

to grow we should expect the moles of ammonia to continue to increase. However, at best the growth is slow and the error of the volume measurements and the natural variation of the sap samples are such that when the rate of ammonia entrance has become slow some uncertainty in the location of the points is to be expected. The simplest interpretation of the flattening is that the concentration of ammonia in the sap has reached a constant level, and that thereafter as the volume increases slowly, ammonia is taken in slowly to keep the concentration constant.

The approach to constant ammonia concentration during ammonia accumulation is also clearly foreshadowed in the curves for Experiment 3 (Fig. 2) and in a curve in a previous paper<sup>8</sup>. But there is little or no flattening of the curves of Experiment 4. This may mean merely that the experiment was terminated before the onset of the flattening. On the other hand it may be connected with the preliminary dark treatment. This point will be the subject of further investigation.

The flattening of the curve suggests the approach to an equilibrium or rather, since we are dealing with a living system, to a "steady state."

For equilibrium we may write that

$$f_o^{\text{NH}_3} [\text{NH}_3]_o = f_i^{\text{NH}_3} [\text{NH}_3]_i$$

where  $f$  is the activity coefficient,  $[\text{NH}_3]$  is the concentration of undissociated ammonia, and  $o$  and  $i$  refer to the sea water and sap respectively.

Assuming<sup>9</sup> that  $f_o^{\text{NH}_3} \approx f_i^{\text{NH}_3}$  the equation reduces to

$$[\text{NH}_3]_o = [\text{NH}_3]_i$$

It can be shown that with certain assumptions the same equation should fit the steady state.

The derivation<sup>10</sup> is as follows. According to Osterhout<sup>5</sup> ammonia may pass through the non-aqueous protoplasm as  $\text{NH}_4\text{X}$  where  $\text{X}$  is the anion of a weak acid elaborated in the non-aqueous layer of the protoplasm. It is assumed that

<sup>8</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 301 (Fig. 3)

<sup>9</sup> Cf. Zscheile, F. P., Jr., *Protoplasma*, 1930, 11, 481

<sup>10</sup> This derivation is given in detail as it will be used as the foundation for calculations in subsequent papers.

the reaction whereby  $\text{NH}_4\lambda$  is formed takes place on the protoplasmic side of the sea water protoplasm or sap-protoplasm interface. We assume that the protoplasm<sup>11</sup> has the structure shown in Fig 4. For purposes of discussion we may neglect the aqueous layer  $W$  and treat the protoplasm as though it consisted of a single non aqueous layer. Now at each interface there is a pair of adjacent unstirred layers, one in the aqueous phase and the other in the non aqueous protoplasmic surface layer. And in each pair of such layers there are thin regions immediately in contact where there is equilibrium across the interface. At the sea water interface the aqueous unstirred layer is designated hereafter as  $op$  and the non aqueous layer as  $po$ . The thin equilibrium layers are called  $cop$  and  $epo$ . At the sap-protoplasm interface the corresponding designations are  $ip$ ,  $pi$ ,  $cip$ , and  $epi$ . (See Fig 4)

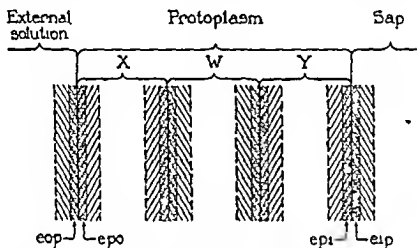


FIG 4 Hypothetical structure of the protoplasm.  $\lambda$  and  $Y$  are non aqueous layers.  $W$  is an aqueous layer. The shaded areas represent unstirred layers. The very narrow stippled bands, bounding each interface are extremely thin layers which are in approximate equilibrium. Some of these are labelled, i.e.  $cop$ ,  $epo$ ,  $epi$ , and  $cip$ .

Now at the steady state if we ignore the small amount of growth which occurs

$$[\text{NH}_4\text{X}]_{epo} = [\text{NH}_4\lambda]_{epi}$$

since in this condition the flux of  $\text{NH}_4\text{X}$ , which is assumed to be the only species carrying ammonia, is zero

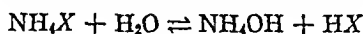
But we assume that at each interface some of the  $\text{NH}_4\text{X}$  formed is transferred to the adjacent aqueous layers so that the following equilibria are set up

$$\left. \begin{aligned} [\text{NH}_4\text{X}]_{epo} &= S_{epo}^{\text{NH}_4\text{X}} [\text{NH}_4\lambda]_{cop} \\ [\text{NH}_4\text{X}]_{epi} &= S_{epi}^{\text{NH}_4\text{X}} [\text{NH}_4\lambda]_{cip} \end{aligned} \right\} \quad (1)$$

where  $S$  is the partition coefficient.

<sup>11</sup> Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, 35, 967

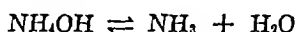
In the aqueous layers we have the following hydrolytic reaction,



for which we can write the thermodynamic mass action equation,

$$K_{\text{hyd}} = \frac{(\text{NH}_4\text{OH})_{\text{cop}}(\text{HX})_{\text{cop}}}{(\text{NH}_4\text{X})_{\text{cop}}(\text{H}_2\text{O})_{\text{cop}}} = \frac{(\text{NH}_4\text{OH})_{\text{cip}}(\text{HX})_{\text{cip}}}{(\text{NH}_4\text{X})_{\text{cip}}(\text{H}_2\text{O})_{\text{cip}}} \quad (2)$$

where parentheses indicate activities But



for which the thermodynamic mass action equation is

$$k_h = \frac{(\text{NH}_3)_{\text{cop}}(\text{H}_2\text{O})_{\text{cop}}}{(\text{NH}_4\text{OH})_{\text{cop}}} \quad (3)$$

Now

$$(\text{HX})_{\text{cop}} = f_{\text{cop}}^{\text{HX}} [\text{HX}]_{\text{cop}} = \frac{f_{\text{cop}}^{\text{HX}} [\text{HX}]_{\text{cpo}}}{S_{\text{cop}}^{\text{HX}}}$$

where square brackets indicate concentrations (A similar set of equations apply to the *cip-cpi* interface)

Whence for the second term of equation (2) we get

$$\begin{aligned} (\text{NH}_4\text{X})_{\text{cop}} &= f_{\text{cop}}^{\text{NH}_4\text{X}} [\text{NH}_4\text{X}]_{\text{cop}} = \frac{f_{\text{cop}}^{\text{NH}_4\text{X}} [\text{NH}_4\text{X}]_{\text{cpo}}}{S_{\text{cop}}^{\text{NH}_4\text{X}}} \\ &= \frac{[\text{NH}_3]_{\text{cop}} [\text{HX}]_{\text{cpo}} f_{\text{cop}}^{\text{HX}} f_{\text{cop}}^{\text{NH}_3}}{K_{\text{hyd}} k_h S_{\text{cop}}^{\text{HX}}} \end{aligned} \quad (4)$$

and for the third term of equation (2)

$$\frac{f_{\text{cip}}^{\text{NH}_4\text{X}} [\text{NH}_4\text{X}]_{\text{cpi}}}{S_{\text{cip}}^{\text{NH}_4\text{X}}} = \frac{[\text{NH}_3]_{\text{cip}} [\text{HX}]_{\text{cpi}} f_{\text{cip}}^{\text{HX}} f_{\text{cip}}^{\text{NH}_3}}{K_{\text{hyd}} k_h S_{\text{cip}}^{\text{HX}}} \quad (4a)$$

or collecting constants

$$[\text{NH}_4\text{X}]_{\text{cpo}} = K'_{\text{coll}} [\text{NH}_3]_{\text{cop}} [\text{HX}]_{\text{cpo}}$$

and

$$[\text{NH}_4\text{X}]_{\text{cpi}} = K''_{\text{coll}} [\text{NH}_3]_{\text{cip}} [\text{HX}]_{\text{cpi}}$$

Or assuming that corresponding activity and partition coefficients are equal in sap and sea water, at the steady state

$$[\text{NH}_3]_{\text{cop}} [\text{HX}]_{\text{cpo}} = [\text{NH}_3]_{\text{cip}} [\text{HX}]_{\text{cpi}} \quad (5)$$

or if there are no concentration gradients of  $[\text{NH}_3]$  across the *op* and *ip* layers, as is almost certainly the case,

$$[\text{NH}_3]_o[\text{HX}]_{ip} = [\text{NH}_3]_i[\text{HA}]_{op} \quad (5a)$$

Now if  $\text{HA}$  is distributed equally throughout the non aqueous protoplasm  $[\text{HX}]_{po} = [\text{HX}]_p$ . For purposes of calculation we may regard  $[\text{HX}]_{po}$  and  $[\text{HX}]_{ip}$  as constant. We then have for the steady state,

$$[\text{NH}_3]_o = [\text{NH}_3]_i \quad (6)$$

If a steady state has been attained we should be able to upset it by changing  $[\text{NH}_3]$ . An experiment to determine if this could be done was carried out by exposing cells which had ceased to accumulate ammonia to (a) sea water containing more ammonia at the normal pH of sea water and (b) to sea water containing the same amount of total ammonia at a higher pH. There were six groups in this part of this experiment.

(a) Light and dark sub groups in which cells from the light and dark groups of Experiment 1 were exposed respectively in normal light and darkness to sea water at the normal pH<sup>1</sup> containing 0.00175 M ammonium chloride. Since this involved no change in conditions these cells were regarded as controls.

(b) Light and dark sub groups in which cells as in (a) were exposed to sea water containing 0.0025 M ammonium chloride at normal pH.

(c) Light and dark sub groups in which cells as in (a) were exposed to sea water containing 0.00175 M ammonium chloride at pH 9.5.

As Table II shows, the concentration of ammonia in the sap increased hardly at all in the control groups, but it did increase decidedly in the others. This suggests that real steady states had been attained in both light and dark groups of Experiment 1 during the exposure of the cells to sea water, at normal pH, containing 0.00175 M ammonium chloride, since in both groups increasing  $[\text{NH}_3]$  caused the concentration of total ammonia in the sap to increase. It is noteworthy that in the dark sub groups (b) and (c) there is a suggestion of an approach to new steady states. This was not the case in the light sub groups, but it may have been that in these cases new steady states would have been attained if it had been possible to continue the experiments further.

We may now compare the steady state concentrations attained in Experiment 1 with values calculated on the assumption that at the steady state  $[\text{NH}_3]_o = [\text{NH}_3]$ .

The normal pH of the sea water is 8.2 and when  $[\text{Am}]_o = \text{total ammonia in sea water} = 0.00175 \text{ M}$ ,  $[\text{NH}_3]_o = 5.9 \times 10^{-5}$ , according to the Henderson-Hasselbalch equation<sup>12</sup>. But the pH of the sap according to the measurements discussed on pages 503 to 508 was about 5.8 at the steady state. At this pH in order for  $[\text{NH}_3]$  to be equal to  $[\text{NH}_3]_o = 5.9 \times 10^{-5}$ ,  $[\text{Am}]$  would have to be approximately 0.4 M.

TABLE II

Light group			Dark group		
Sub group	Days	Concentration ammonia in sap M	Sub group	Days	Concentration ammonia in sap M
(a) Control	0	0.1329	(a) Control	0	0.0494
	2	0.1343		2	0.0512
	5	0.1400		5	0.0499
				12	0.0532
(b)	0	0.1329	(b)	0	0.0494
	2	0.1655		5	0.0704
	5	0.2072		12	0.0729
(c)	0	0.1329	(c)	0	0.0494
	2	0.1500		2	0.0650
	5	0.1815		5	0.0688

Actually it was about 0.05 M. In the light group  $[\text{Am}]_i = 0.13 \text{ M}$  which is 6/10 of the theoretical value of  $[\text{NH}_3]_o = 5.9 \times 10^{-5}$  when  $\text{pH}_o$  is taken as 8.2. As a matter of fact it is almost certain that during the period of illumination at least,  $\text{pH}_{\text{sap}}$  is greater than  $\text{pH}_o$ , since by photosynthesis  $\text{CO}_2$  is removed from the sea water, and although with a large body of sea water in proportion to the cell volume, as was used in our experiments, this effect does not show up in the bulk of sea water, some increase of pH in the unstirred layer of

<sup>12</sup> In this calculation  $\text{pK}'_b$  was taken as 4.34. The reason for this choice is given in a previous paper (Jacques, A. G., *J. Gen. Physiol.*, 1935-36, 19, 397).

sea water in the cellulose wall adjacent to the protoplasm seems inevitable in light

Crozier<sup>12</sup> has shown that in aquaria the photosynthesis of *Valonia* may raise the pH to 9.5 in sunlight and this agrees with our results when the volume of cells to sea water is not too great. Apparently at 9.5 the precipitation of calcium or magnesium carbonates or both serves to buffer the system somewhat by removing  $\text{CO}_3^{2-}$  ion so that 9.5 is the limit to which  $\text{pH}_{\text{cop}}$  can rise during illumination.

At this pH the maximum value of  $[\text{NH}_3]_{\text{cop}}$  neglecting loss of  $\text{NH}_3$  from cop to o by diffusion would be  $7.0 \times 10^{-4}$ .

But pH according to our measurements, in the light group, was 6.10 at the end of the experiment. Hence  $[\text{Am}]_i$  should be 2.5 M in order for  $[\text{NH}_3]_i$  to be equal to  $[\text{NH}_3]_{\text{cop}} = 7.0 \times 10^{-4}$ . The steady state value of  $[\text{NH}_3]_i$  corresponding to  $[\text{NH}_3]_o$  in normal light must be somewhere between 0.21 M and 2.5 M and in any case it is greater than 0.13 M, the value found.

These calculations are only approximate since they depend on the somewhat arbitrarily selected value of 4.34 for  $\text{pK}'_i$ . However, calculating for the dark group where complications due to photosynthesis are absent the pH which the sap would have to have in order to make  $[\text{NH}_3]_o = [\text{NH}_3]_i$  when  $\text{pH}_o = 8.2$  and  $\text{pH}_i = 5.8$ , we get  $\text{pH} = 6.7$ . The actual value was 5.80 and the discrepancy can scarcely be explained away even by making all possible allowances for error in the pH measurements and error in the selection of the value for  $\text{pK}'_i$ .

It will be recalled (p. 512) that the conclusion that  $[\text{NH}_3] = [\text{NH}_3]$  at the steady state is based on the assumptions that (a) corresponding activity coefficients in sap and sea water are equal, (b) corresponding partition coefficients in sap and sea water are equal, and (c)  $[\text{HX}]_{\text{apo}} = [\text{HX}]_{\text{epi}}$ .

The first is almost certainly valid since the ionic strengths of sap and sea water are not far apart. In general we have assumed that (b) also is valid, but our information in this respect is vague. But in regard to (c) it is not improbable that  $[\text{HX}]_{\text{epi}} > [\text{HX}]_{\text{apo}}$ . Such a situation could arise if HX is elaborated only at the sap protoplasm

<sup>12</sup> Crozier W. J. *J. Gen. Physiol.*, 1918-19, 1, 581



interface In this case the equation governing the steady state is equation (5a) (p 513), i.e.,

$$[\text{NH}_3]_o [\text{HX}]_{cpo} = [\text{NH}_3]_i [\text{HX}]_{cpi}$$

Now according to the calculation for the dark group (p 514), where complications due to photosynthesis are absent,  $[\text{Am}]_i$  is only 1/8 of what it should be in order for  $[\text{NH}_3]_i$  to equal  $[\text{NH}_3]_o$ . But if  $[\text{HX}]_{cpo}$  is only 1/8  $[\text{HX}]_{cpi}$ , equation (5a) will be satisfied, i.e., the ratio  $[\text{HX}]_{cpo} - [\text{HX}]_{cpi} = 0.125$

It is assumed that all the ammonia passing through the protoplasm travels as  $\text{NH}_4\text{X}$  whence the flux for cells with surface of unit area and thickness is given by

$$\frac{d[\text{Am}]}{dt} = D^{\text{NH}_4\text{X}} \{ [\text{NH}_4\text{X}]_{cpo} - [\text{NH}_4\text{X}]_{cpi} \} \quad (7)$$

where  $[\text{Am}]$  means concentration of ammonia  $D$  is a constant for the movement of  $\text{NH}_4\text{X}$  in the protoplasm which is the analogue of a diffusion constant

When  $[\text{HX}]_{cpo}$  is considered to be equal to  $[\text{HX}]_{cpi}$  and constant this reduces to

$$\frac{d[\text{Am}]}{dt} = D^{\text{NH}_4\text{X}} K'_{\text{coll}} [\text{HX}]_{cpo} \{ [\text{NH}_3]_{cpo} - [\text{NH}_3]_{cpi} \} \quad (8)$$

$$K'_{\text{coll}} = \frac{S_{cop}^{\text{NH}_4\text{X}}}{S_{cop}^{\text{H}_2\text{O}}} \frac{f_{cop}^{\text{H}_2\text{O}} f_{cop}^{\text{NH}_3}}{f_{cop}^{\text{NH}_4\text{X}}} \frac{1}{K_{\text{hyd}} k_h} \quad (8a)$$

We now proceed to calculate the permeability constants from equation (8) Putting this in a familiar form<sup>14</sup> for simplicity, we have,

$$\frac{dx}{dt} = P'(a - x) \quad (9)$$

where  $a = [\text{NH}_3]_{cpo}$ ,  $x = [\text{NH}_3]_{cpi}$ , and  $P'$  contains all the constants of equation (8) On integration

$$P' = \frac{2.3}{t} \log \frac{a}{a - x} \quad (10)$$

<sup>14</sup> This is, of course, only an approximation in many cases, cf Jacobs, M. H., *Ergebn Biol*, 1935, 12, 1 According to this equation the rate is directly proportional to  $\text{NH}_3_o - \text{NH}_3_i$ . But this is not necessarily true when the initial rates with different concentrations of  $\text{NH}_3_o$  are compared (see footnote 5)

In the present case we have first applied equation (10) to the dark group of Experiment 1 (Table I)  $a$  has been calculated on the assumption that the  $\text{pH}_o = \text{pH}_{op} = 8.2$  and  $[\text{Am}]_o = [\text{Am}]_p$ , whence  $[\text{NH}_3]_{op} = a = 5.9 \times 10^{-6}$  (see p 514)  $x$  has been calculated from  $[\text{NH}_3]_p$  on the assumption that  $\text{pH}_{op} = \text{pH}_i = 5.90$ . The result is given in Table I under  $P'_D$ . It will be seen that this "constant" decreases steadily with time.

If  $[\text{HX}]_{op} \approx [\text{HX}]_{ip}$ , we must put

$$\frac{d[\text{Am}]}{dt} = D^{\text{NH}_4\text{X}} K'_{\text{coll}} \{[\text{NH}_3]_{op} [\text{HX}]_{op} - [\text{NH}_3]_{ip} [\text{HX}]_{ip}\}$$

Putting this in the simplified form

$$\frac{dx}{dt} = P'' \{ba - cx\}$$

where  $b = [\text{HX}]_{op}$  and  $c = [\text{HX}]_p$ . Since we do not know the absolute values of either  $b$  or  $c$  we multiply the right hand by  $c/c$  to get

$$\frac{dx}{dt} = P' \left( \frac{b}{c} a - x \right)$$

where  $P''' = cP''$ . On integration we get

$$P' = \frac{2.3}{t} \log \frac{\frac{b}{c} a}{\frac{b}{c} a - x} \quad (11)$$

This equation has been used to calculate  $P'''$ . The most natural assumption to make in calculating the coefficient  $b/c$  is that at the end of the experiment when the steady state was attained

$$b/c [\text{NH}_3] = [\text{NH}_3]_i$$

Assuming that  $\text{pH}_i = 5.90$  and  $[\text{Am}]_i$  at the steady state was  $0.0518$ ,  $[\text{NH}_3]_i = 9.0 \times 10^{-6}$  and this value was used to calculate the permeability constant for the dark group. By trial we found that the value  $8.83 \times 10^{-6}$  for  $b/c [\text{NH}_3] = [\text{NH}_3]_i$ , gave a slightly better series of values for the permeability constant. These values are

<sup>15</sup> We may not assume that at the steady state  $b/c a < x$ , but the slight correction from  $9.0 \times 10^{-6}$  to  $8.83 \times 10^{-6}$  is within the limits of the natural variations of the cells.

given as  $P_D''$  in Table I. Although the values are erratic there is no definite trend and the deviations may be associated with faulty sampling of the sap and errors in analysis.

On using the value  $1.0 \times 10^{-5}$  for  $b/c$   $[\text{NH}_3]_0$ , the values of the permeability constant had a marked trend. Hence we conclude that  $8.83 \times 10^{-6}$  is a significant value<sup>16</sup>. And the ratio  $b/c$  is therefore approximately

$$\frac{8.83 \times 10^{-6}}{5.9 \times 10^{-5}} \simeq 0.15$$

In the light group, Experiment 1 (Table I), we may not assume that  $\text{pH}_{\text{cop}} = \text{pH}_0$  at least not during illumination. According to Crozier<sup>13</sup> the maximum pH which can be expected due to the photosynthetic removal of  $\text{CO}_2$  from the sea water is 9.5. Using this value for  $\text{pH}_{\text{cop}}$ ,  $[\text{NH}_3]_{\text{cop}} = 1.1 \times 10^{-4}$ . But the cells were, in our experiments, illuminated not more than half the time due to the onset of darkness, and in the dark period  $\text{pH}_{\text{cop}}$  may be taken as equal to  $\text{pH}_0 = 8.2$ , whence  $[\text{NH}_3]_0 = 5.9 \times 10^{-5}$ . The average of these two values  $\simeq 3.85 \times 10^{-4}$  has been taken as the value for  $a$  in calculating  $P'_L$  from equation (10) and  $\alpha$  has been calculated on the basis that  $\text{pH}_i = 6.15$ . As Table I shows,  $P'_L$  calculated in this way has an obvious drift.

We now apply equation (11) using  $b/c = 0.15$  as in the dark group.  $b/c \cdot a$  then equals  $5.78 \times 10^{-5} \text{ M}$ .

The permeability constant has been calculated on this basis. It was found to be nearly without trend but with a slight upward drift at the end. A slightly better constant was obtained by using for  $b/c \cdot a$ ,  $5.9 \times 10^{-5}$ . This is  $P_L''$  in Table I.

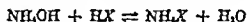
Since by using the factor  $b/c = 0.15$  we obtain fairly satisfactory permeability constants we assume that this ratio may have some physical significance. Provisionally we suppose that it is the ratio of  $[\text{HX}]_{\text{cp1}}/[\text{HX}]_{\text{cp0}}$ . The actual concentration of each is unknown.

<sup>16</sup> It might be suggested that since the pH value of the sap used in these calculations, *viz.* 5.90, is a compromise value, being the apparent average value of the pH during the entrance of ammonia, that we could correct  $[\text{NH}_3]_0$  from  $9.0 \times 10^{-6}$  to  $8.83 \times 10^{-6}$  by assuming a slightly lower value for  $\text{pH}_i$ . But if this corrected value is used in calculating  $\alpha$  the constants are the same as those obtained by using  $9.0 \times 10^{-6}$  and  $\text{pH}_i = 5.99$ .

If  $b/c$  has the significance suggested at the steady state  $x$  should be equal to  $b/c a$ . In the dark group  $x_{\infty}$ , where  $c$  signifies the steady state, was found to be equal to  $9 \times 10^{-6}$ , but  $b/c a$  was taken as  $8.83 \times 10^{-6}$ . This slight discrepancy is of no importance. In the light group conditions are more complicated, because owing to photosynthesis  $a$  is not well known. But by taking  $a$  as the probable average value of  $[\text{NH}_3]_{\infty}$  in light and in darkness a value for  $b/c a = 5.9 \times 10^{-5}$  was obtained. This value gave a good series of values for the constant but  $x$  in the light was only  $4.1 \times 10^{-5}$ . Although the two values are of the same order there is some discrepancy here not yet explained.<sup>17</sup>

It appears that  $P_D'''$  is about 3 times  $P_L'''$ . Assuming that  $P_D'' = P_L''$  we may suppose that  $[\text{HX}]_{\text{ext}} = c$  is 3 times as great in the dark. This seems possible for we must remember that some of the  $\text{HX}$  is being lost to the sea water and this loss may well be much greater in light than in darkness due to the higher pH at the sea water protoplasm interface when the cell is photosynthesizing actively. We assume that  $[\text{HX}]_{\text{ext}}$  is also 3 times as great in the dark.

Hitherto we have assumed that the reaction is a simple reversible neutralization



But it might be much more complex than this. This will be discussed in a forthcoming paper.

#### SUMMARY

The accumulation of ammonia takes place more rapidly in light than in darkness. The accumulation appears to go on until a steady state is attained. The steady state concentration of ammonia in the sap is about twice as great in light as in darkness. Both effects are possibly due to the fact that the external pH (and hence the concentration of undissociated ammonia) outside is raised by photosynthesis.

Certain "permeability constants" have been calculated. These

<sup>17</sup> A slight uncertainty in the determination of  $\text{pH}_{\text{ext}}$  at the steady state would account for the discrepancy but since  $x$  and  $x_{\infty}$  are calculated from the same value of  $\text{pH}$  the calculated value of the constant would again show large deviations if  $\text{pH}_{\text{ext}}$  were increased enough to make  $x = 5.9 \times 10^{-5}$ .

indicate that the rate is proportional to the concentration gradient across the protoplasm of  $\text{NH}_4\text{X}$  which is formed by the interaction of  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  and  $\text{HX}$ , an acid elaborated in the protoplasm. The results are interpreted to mean that  $\text{HX}$  is produced only at the sap-protoplasm interface and that on the average its concentration there is about 7 times as great as at the sea water-protoplasm interface. This ratio of  $\text{HX}$  at the two surfaces also explains why the concentration of undissociated ammonia in the steady state is about 7 times as great in the sea water as in the sap. The permeability constant  $P'''$  appears to be greater in the dark. This is possibly associated with an increase in the concentration of  $\text{HX}$  at both interfaces, the ratio at the two surfaces, however, remaining about the same.

The pH of sap has been determined by a new method which avoids the loss of gas ( $\text{CO}_2$ ), an important source of error. The results indicate that the pH rises during accumulation but the extent of this rise is smaller than has hitherto been supposed.

As in previous experiments, the entering ammonia displaced a practically equivalent amount of potassium from the sap and the sodium concentration remained fairly constant.

It seems probable that the pH increase is due to the entrance of small amounts of  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  in excess of the potassium lost as a base.

## THE KINETICS OF PENETRATION

### XVII THE EXIT OF AMMONIA IN LIGHT AND DARKNESS

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In a previous paper<sup>1</sup> dealing with the exit of ammonia from cells of *Valonia macrophysa*, Kütz, it was suggested that the rate may be greater in light than in darkness and that the exit is preceded by an induction period. In the present paper these points have been studied in more detail. And at the same time the pH changes of the sap during the exit have been studied by a new method.<sup>2</sup>

In this paper, "exposure to light" means exposure to the normal alternation of daylight and darkness in the laboratory, "dark" means continuous darkness.

#### EXPERIMENTAL

These experiments were carried out in Bermuda in the winter of 1937-38 at the Bermuda Biological Station.

Cells which had been allowed to accumulate ammonia in the light were divided into two groups and were exposed to a flow of normal sea water, which is nearly ammonia free, one group in darkness and the other in light.

The cells were exposed in glass jars in the light groups and in darkened glass jars or porcelain jars in the dark groups as described in the previous paper. The jars were closed with two layers of heavy opaque rubber sheeting through which were thrust two glass tubes for the entrance and exit of the flow of sea water. There may have been some slight leakage of light into the dark jars from this cause but this must have been very small since the tubes themselves were blackened.

<sup>1</sup> Jacques, A G, *J Gen Physiol* 1937-38 21, 775

Ammonia means, as in previous papers,  $\text{NH}_3 + \text{NH}_4\text{OH} + \text{NH}_4 \text{ ion}$  : e the total amount found by analysis

<sup>2</sup> Jacques A G, *J Gen Physiol*, 1938-39 22, 501

Volumes were determined on small groups of cells assumed to be characteristic of the whole groups by a method previously described<sup>4</sup>. Analyses for potassium, sodium, and ammonia were carried out as described in a recent paper. For the pH of the sap we used the new technique recently described<sup>1</sup> in which the loss of gas, chiefly CO<sub>2</sub>, from the extracted sap is avoided.

TABLE I

*Exit of Ammonia from Valonia in Light and Dark (Experiment 1)*

Days	Volume	Concentration			Moles $\times 10^4$		
		Ammonia	Potassium	Sodium	Ammonia	Potassium	Sodium
Light group							
	cc	M	M	M			
0	1 72	0 1260	0 3617	0 1426	2 167	6 221	2 452
2	1 73	0 1270	0 3547	0 1397	2 197	6 136	2 416
5	1 76	0 1188	0 3576	0 1432	2 090	6 294	2 520
10	1 76	0 1186	0 3699	0 1384	1 987	6 520	2 436
15	1 78	0 0863	0 3668	0 1587	1 536	6 529	2 824
21	1 82	0 0739	0 3568	0 1783	1 345	6 493	3 235
26	1 86	0 0615	0 3336	0 2195	1 144	6 204	4 072
Dark group							
	Adjusted volume*						
0	1 72	0 1260	0 3617	0 1426	2 167	6 221	2 452
2	1 73	0 1279	0 3583	0 1342	2 212	6 198	2 311
5	1 67	0 1345	0 3587	0 1292	2 246	5 990	2 158
10	1 70	0 1154	0 3662	0 1514	1 962	6 225	2 563
15	1 70	0 1168	0 3534	0 1539	1 984	6 007	2 616
21	1 73	0 1025	0 3335	0 1738	1 906	5 769	3 007
26	1 78	0 0905	0 3433	0 1933	1 738	6 110	3 441

\* Adjusted by multiplying the measured volume in the dark group by 1.72 - 1.85 so as to make it possible to start light and dark group "moles" curves at the same zero point.

Three experiments were performed.

*Experiment 1*—The cells were exposed to sea water containing 0.0025 M ammonium chloride for 14 days in light. Then they were divided into two groups, one in darkness and the other in light, and kept in flowing normal (nearly ammonia-free) sea water for 26 days.

<sup>4</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, 15, 537.

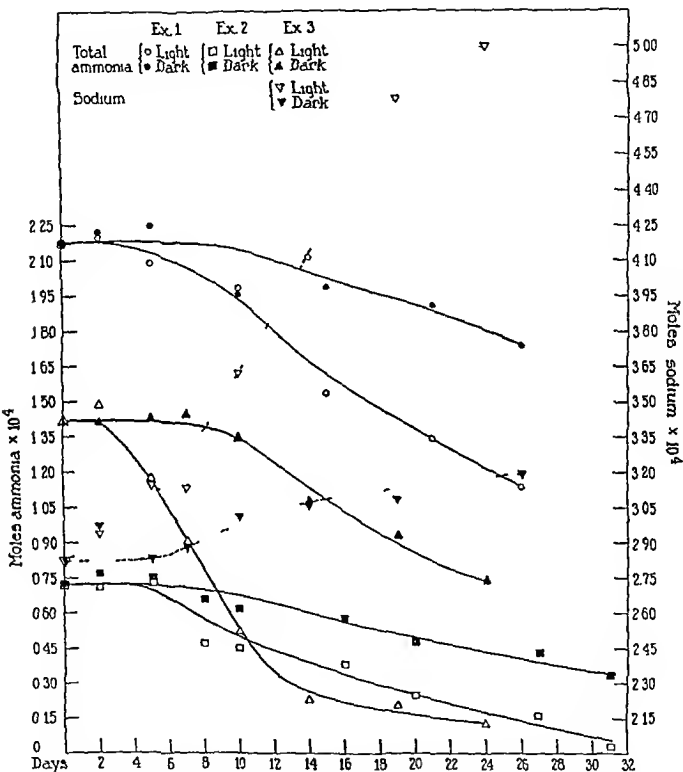


FIG. 1 The exit of ammonia in light and darkness Experiments 1, 2, and 3, and the entrance of sodium in light and darkness in Experiment 3. The curves are drawn free hand to give an approximate fit.



in light The results of this experiment are given in Table I and in Fig 1

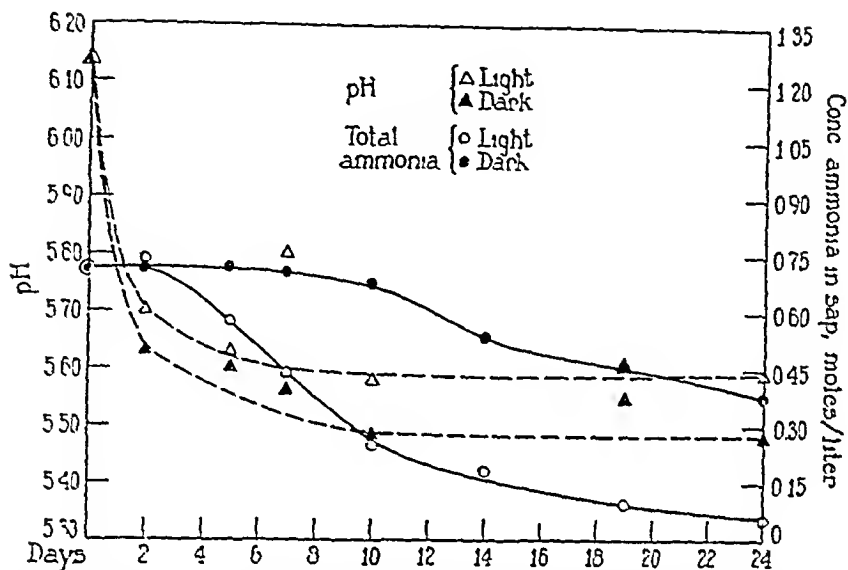


FIG 2 Comparison of the decrease of ammonia concentration and the change of pH of the sap in light and darkness The curves are drawn free-hand to give an approximate fit

TABLE II  
Initial and Final Concentrations

Experiment		Stage 1 Before accu- mulation	Stage 2 At end of ac- cumulation	At end of exit	
				Light group	Dark group
1	Ammonia	0 0005	0 1260	0 0615	0 0905
	Potassium	0 4953	0 3617	0 3336	0 3433
	Sodium	0 1348	0 1335	0 2195	0 1933
2	Ammonia	0 0005	0 0615	0 0017	0 0245
	Potassium	0 4954	0 4464	0 4427	0 4106
	Sodium	0 1297	0 1304	0 1836	0 1906
3	Ammonia	0 0007	0 0707	0 0057	0 0372
	Potassium	0 4957	0 4274	0 3953	0 4130
	Sodium	0 1430	0 1390	0 2210	0 1596

Experiment 2 —The cells were first allowed to accumulate ammonia from sea water containing 0 00100 M ammonium chloride for 13 days

in light After this some were exposed in light and others in darkness to normal sea water for 31 days The results are given in Fig 1

*Experiment 3*—The cells were allowed to accumulate ammonia from sea water containing 0.00175 M ammonium chloride for 21 days in light Afterwards some were exposed in light and others in darkness to normal sea water for 24 days The results are given in Figs 1 and 2 which show the decrease in concentration of ammonia for both light and dark groups during the exposure to normal sea water in comparison with the pH change during exit For comparison see also Fig 3 of a previous paper<sup>3</sup> (plotted on the same scale) showing how the pH behaves during accumulation

Table II gives a summary of the behavior of ammonia, potassium, and sodium, in Experiments 1, 2, and 3, during the accumulation and exit of ammonia

#### DISCUSSION OF RESULTS

It appears that during exposure to sea water nearly free from ammonia the exit of ammonia is in all cases preceded by an "induction period" during which the concentration of ammonia either remains constant or the decrease is so slow that it is masked by the natural variations among the cells The induction period is longer in the dark, and when at length the exit of ammonia occurs at a measurable rate, the loss proceeds more rapidly in the light than in the dark Eventually, however, when the residual concentration of ammonia in the sap has reached a low value the rate of exit decreases again Thus the curve has an S shape The S shape, however, is not clearly apparent in the curves for the exit in the dark, but this may well be because the rate of exit was slow with the result that either the S shape was masked by the natural variations among the cells or it had not appeared by the time the experiments were concluded

As in the previous experiments<sup>3</sup> the ammonia lost was replaced largely by sodium A precise idea is hard to get because of the natural variation in the K/Na ratio of different groups of cells However, we may say with a fair degree of certainty that in 5 of the 6 cases studied when ammonia left the sap the sodium concentration increased and the potassium concentration remained quite constant It is not always possible to equate the moles of sodium gained to the

moles of ammonium lost This is partly owing to the natural variations, and partly perhaps to a tendency for the total cation concentration of the sap to decrease during exit <sup>5</sup> This may mean that ammonia is lost at a slightly greater rate than sodium is gained In one experiment, the light group of Experiment 6, the cell gained moles of potassium also during the exit of ammonia

However, in this experiment a new situation was encountered, since in the last 11 days of the 31 day exposure to normal sea water, the rate of growth was much greater than during the first 20 days Thus in the first 20 days the increase in volume was only about 6 per cent, but in the 11 day period it was about 20 per cent This seems to be chiefly because during the last part of the experiment the cells commenced to bud actively The sap samples came from both buds and parent cells, since only by including both could we get fair values for the moles of ammonia in the sap Both buds and parent cells were necessarily included in the volume measurements But the bud cells not having been exposed to the sea water containing ammonia may not have had any ammonia in them unless they took it from the mother cell Perhaps such bud cells are able to take up potassium preferentially as do cells which have not been exposed to ammonia Their inclusion in the sample of sap would therefore tend to raise the potassium concentration of the sample, hence it is not settled by this experiment whether cells which have once accumulated ammonia can again take up potassium Even in the previous experiment<sup>1</sup> where we seemed to have part of the ammonia replaced by potassium, it is not impossible that the result may have been influenced by the inclusion of buds

In any case we may say that when ammonia leaves the cell it tends to be replaced by sodium alone

In the present series of experiments the cells were tested for their ability to reaccumulate ammonia after some of the ammonia had been replaced by sodium In some cases two accumulations and two partial removals of ammonia were carried out In all cases the cells were able to reaccumulate ammonia Some of these experiments are described below

<sup>5</sup> Conversely when ammonia is being accumulated actively the total cation concentration generally rises above normal

In one of them cells from the light group of Experiment 1, Table I, were divided into two sub groups, and exposed to sea water containing 0.0025 M ammonia, for 8 days (a) in light, and (b) in darkness. The analyses follow

	Initial (a) and (b)	(a) after 8 days (light)	(b) after 8 days (dark)
	M	M	M
Ammonia	0.0615	0.1565	0.0628
Potassium	0.3336	0.2448	0.3271
Sodium	0.2195	0.2295	0.2185
Total	0.6146	0.6308	0.6084

In this experiment the cells took up ammonia rapidly in the light, and while the concentration of ammonia was rising by 0.0950 M, the concentration of potassium decreased by 0.0888 M. In the dark sub group, however, all concentrations remained practically unchanged.

The cells of the (b) sub group were then exposed to the same ammoniated sea water in light, and the cells of (a) sub group to a flow of normal nearly ammonia free sea water in light, both for 28 days. The following results were obtained

	(a) 28 days in normal sea water In light	(b) 28 days in sea water containing 0.0025 M ammonia In light
	M	M
Ammonia	0.0257	0.1957
Potassium	0.3308	0.2509
Sodium	0.2601	0.1890
Total	0.6166	0.6356

In the (a) part of the experiment (in normal sea water) the ammonia concentration decreased by 0.1308 M, but the sodium concentration increased only by 0.0306 M, while the potassium concentration increased by 0.0860 M. This is fairly good evidence that in some cases at least the ammonia lost can be replaced by potassium. The increase in the potassium concentration seems too great to be due to faulty sampling, although in this case errors due to this source were probably aggravated because only a few cells were available at this stage for each sample.

In the (*b*) sub-group the ammonia concentration increased by 0.1329 M, but the potassium decreased by only 0.0762 M. This may be due in part to faulty sampling, but undoubtedly another factor is the marked increase in the total cation concentration which occurred during the reaccumulation. The excess concentration may well be ammonia accumulated without the loss of a corresponding amount of potassium.

Since the (*b*) group cells were able to accumulate ammonia in the light we may suppose that they failed to do so in the dark because the internal ammonia concentration was already at the steady state equilibrium corresponding to the external concentration of ammonia in the dark. In this connection it may be said that in Experiment 1 of a previous paper,<sup>3</sup> where the cells were also exposed to sea water containing 0.0025 M ammonia in the dark, the ammonia concentration in the sap ceased to rise when it reached 0.069 M, which is within reasonable distance of the value 0.063 M found in the present experiment.

Finally the remaining cells of sub-group (*a*) were again exposed to 0.0025 M ammonia sea water, for 5 days in light. The result was as follows:

	Initial	After 5 days in sea water containing 0.0025 M ammonia sea water
	M	M
Ammonia	0.0257	0.1014
Potassium	0.3308	0.2946
Sodium	0.2601	0.2476
Total	0.6166	0.6436

In this case the concentration of ammonia rose 0.0757 M but the potassium concentration decreased by only 0.0362 M. Most of the difference is accounted for by the rise in the total cation concentration by 0.0270 M.

In another experiment the cells were first exposed to sea water containing 0.001 M ammonium chloride for 13 days. They were then transferred to running normal sea water for 33 days. Then they were exposed to sea water containing 0.00175 M ammonium chloride for 15 days, and finally again to running normal sea water for 12 days. All these exposures were in light. The results were as follows:

	Initial	Exposed to 0.001 M ammonia S W 13 days	Exposed to normal S W 33 days	Exposed to 0.00175 M ammonia S W 15 days	Exposed to normal S W 12 days
	M	M	M	M	M
Ammonia	0.0005	0.0508	0.0045	0.1700	0.0948
Potassium	0.4949	0.4462	—	0.2843	0.2616
Sodium	0.1297	0.1484	—	0.1626	0.2506
Total	0.6251	0.6454	—	0.6169	0.6070

Although the experiment lasted 73 days, those cells which were allowed to survive to the end of it, after two exposures to sea waters containing ammonia, and two partial removals of ammonia in normal sea water, were apparently uninjured. In the course of the experiment K/Na was reduced from 3.8 to 1.

We may now consider the results of the pH measurements. As Fig. 2 shows, there is apparently very little correlation between the decrease in the pH and the loss of ammonia. Thus in both the light and dark groups the pH dropped promptly from above 6 to between 5.60–5.70. In the case of the light group this was nearly the entire drop during the experiment. In the dark group there was a further drop of perhaps 0.15 pH unit on the average, but this appeared to occur before the end of the induction period. These results reinforce a conclusion drawn in a previous paper<sup>3</sup> that even when there is considerable ammonia in the sap the buffer system of the sap is not an ammonium salt ammonia one which tends to maintain the pH above that of the normal sodium (potassium) bicarbonate  $\text{CO}_2$  system. Instead, the change in pH during ammonia accumulation seems to be connected with the gain of a little free ammonia or ammonium hydroxide in excess of the potassium lost, and in exit with the loss of a little more ammonia or ammonium hydroxide in excess of the sodium gained.

It might be suggested that since exit is preceded by an induction period there is no evidence that any basic ammonia is lost as soon as the cell is exposed to ammonia free sea water. However, only a little ammonia would have to be lost to produce the fall in pH observed, since the buffer capacity of the sap is very low. Such an amount might be lost without appearing in the analyses since it could be smaller than the natural variations among the cells. The induction

period instead of being a period of no loss might be a period during which the rate of loss is so small that it is masked by natural variations

The greater decrease in pH in the dark group might be due to the fact that in the dark none of the  $\text{CO}_2$  produced by the protoplasm is used up photosynthetically <sup>6</sup>

According to a previous paper<sup>3</sup> the rate of accumulation is given by the formula<sup>7</sup>

$$\frac{d[\text{Am}]}{dt} = D^{\text{NH}_4^+} K_{\text{coll}}''' ([\text{HX}]_{epo} [\text{NH}_3]_{eop} - [\text{HX}]_{epi} [\text{NH}_3]_{eip})$$

where [Am] is the ammonia in the sap, square brackets represent concentrations, and HX is a weak acid elaborated by the protoplasm *eop* and *epo* refer respectively to the adjacent equilibrium layers in sea water and protoplasm where all species are in equilibrium across the interface, and *eip* and *epi* refer to a corresponding pair of adjacent layers at the sap-protoplasm interface. In the light the pH in the *eop* layer, owing to the photosynthetic removal of  $\text{CO}_2$ , is greater than in darkness, consequently  $[\text{NH}_3]_{eop}$ , the concentration of undissociated ammonia in *eop*, is greater and so the rate of accumulation is greater.

But when we place the cells in normal sea water which is almost ammonia-free, the direction of the gradient represented by the term inside the brackets is reversed. Consequently ammonia should leave the cell and there is no apparent reason for an induction period. Of course, the pH of the sap drops as soon as the cell is exposed to the ammonia-free sea water and this would reduce  $[\text{NH}_3]_{eip}$ . But even so the direction of the gradient is still outward.

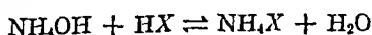
Before going on to a discussion of possible reasons for the induction period we may inquire if injury plays any part.

<sup>6</sup> We suppose that photosynthesis occurs chiefly at the sea water-protoplasm interface but this must increase the gradient of  $\text{CO}_2$  from the sap to the sea water and  $\text{CO}_2$  will be lost faster by the sap.

<sup>7</sup> The basic equation is

$$\frac{d[\text{Am}]}{dt} = D^{\text{NH}_4^+} \{[\text{NH}_4^+]_{epo} - [\text{NH}_4^+]_{epi}\}$$

which is the equation for the diffusion of the species  $\text{NH}_4^+$  a molecule produced by the reversible reaction



We might suppose that the cell has a definite trapping mechanism, capable of retaining ammonia and possibly other cations in the sap indefinitely as long as the mechanism is unimpaired. From Fig 1, it appears that in some cases not only did the concentration fail to increase during the last days of accumulation, but there is a suggestion that it decreased slightly. Although we consider the apparent decreases as due to natural variations among the cells, it might be that ammonia has started to come out as the result of injury. On this basis we should not expect an induction period. On the other hand, we might suppose that the cell is normal at the start of the washing out process and retains the ammonia until injury occurs. But if the cell is not injured during exposure to sea water containing ammonia we should not expect injury to occur when it is placed in normal sea water.

The displacement of ammonia by sodium instead of by potassium which is observed, would be expected if the protoplasm were injured so as to be permeable to all the molecular species of the sap and sea water. But as this would take place by simple diffusion there is no reason why it should take place more rapidly in the light as it undoubtedly does. Nor should we expect injury to set in sooner in the light. Moreover we should expect potassium to be lost also, and probably more rapidly than ammonia, for the concentration of potassium chloride in the sap was in all cases much greater than that of the ammonium chloride, but their diffusion coefficients are roughly equal.<sup>8</sup>

These arguments lead to one conclusion, namely that injury is not an important factor in the exit of ammonia.

In looking for reasons for the induction period we come to the possibility that ammonia is transferred between the sap and sea water by some carrier other than the  $\text{NH}_4\text{X}$  discussed in the previous paper.<sup>2, 7</sup>

The obvious possibilities are undissociated ammonia, the derivatives of carbon dioxide and ammonia, such as ammonium carbonate, ammonium bicarbonate, and ammonium carbamate, and ammonium chloride. At the start of the exit experiments there should be an outwardly directed gradient for all of these species. But there is no

<sup>8</sup> International Critical Tables McGraw Hill Book Company, Inc. New York 1929, 5, 65 68



apparent reason why there should be an induction period in the exit of any of them. Indeed at the very beginning, before the pH drop in the sap has occurred, we should expect the greatest rate of exit and not an induction period. The decrease in the pH observed at the start of the exposure to normal sea water would be expected in all but the case of  $\text{NH}_4\text{Cl}$  to decrease the rate of movement of the species, and in the latter case there should be no effect of pH at all.

The rate of exit in the case of undissociated ammonia would be given by the equation,

$$\frac{d \text{NH}_3}{dt} = K_1 ([\text{NH}_3]_i - [\text{NH}_3]_o) \quad (a)$$

for ammonium bicarbonate,

$$\frac{d \text{NH}_3}{dt} = K_2 ([\text{NH}_4^+], [\text{HCO}_3^-]_i - [\text{NH}_4^+]_o [\text{HCO}_3^-]_o) \quad (b)$$

for ammonium carbamate,

$$\frac{d \text{NH}_3}{dt} = K_3 ([\text{NH}_4^+], [\text{NH}_2\text{COO}^-]_i - [\text{NH}_4^+]_o [\text{NH}_2\text{COO}^-]_o) \quad (c)$$

for ammonium carbonate,

$$\frac{d \text{NH}_3}{dt} = K_4 ([\text{NH}_4^+], [\text{NH}_4^+], [\text{CO}_3^{=}]_i - [\text{NH}_4^+]_o [\text{NH}_4^+]_o [\text{CO}_3^{=}]_o) \quad (d)$$

and for ammonium chloride,

$$\frac{d \text{NH}_3}{dt} = K_5 ([\text{NH}_4^+], [\text{Cl}^-]_i - [\text{NH}_4^+]_o [\text{Cl}^-]_o) \quad (e)$$

A drop in the pH of the sap, should decrease (a) by decreasing  $[\text{NH}_3]_i$ , and also (b), (c), and (d) by decreasing the concentration of the anion. There should be little or no change in (e) because the concentration of ammonium is comparatively great.

We may now consider whether ammonia may not be transported from the sap by some other species than a simple salt. The slow formation<sup>9</sup> of this substance might account for the induction period. A possible species might be urea, which does as a matter of fact occur rather widely in plants.<sup>10</sup>

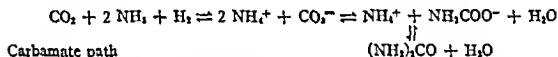
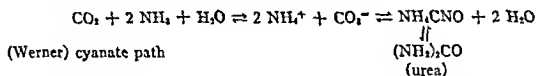
<sup>9</sup> The formation of carbamate from carbonate, may be a comparatively slow reaction, at great dilution (Faurholt, C, *J. chim. phys.*, 1925, 22, 1) but in the pH range of the sap, according to Faurholt's figures, the proportion of carbamate which can exist in equilibrium with other  $\text{CO}_2\text{-NH}_3$  species is almost infinitely small.

<sup>10</sup> For a list of occurrences see *Handbuch der Pflanzenanalyse* (G. Klein), III IV/2, p. 224, J. Springer, Vienna, 1933.

The establishment of the equilibrium in solution between urea and other  $\text{CO}_2\text{-NH}_3$  species is slow even at high temperatures, but with catalysts it can set up rapidly even at ordinary temperatures. Urease which also occurs in plants is a catalyst for the reaction, which seems to be reversible in its effects. That is to say in a urea solution, urease will bring about decomposition until the equilibrium point is reached, or in a solution of 'ammonium carbonate'<sup>11</sup> it will bring about the synthesis of urea.<sup>12</sup>

The nature of the reaction is in doubt. Werner<sup>13</sup> believes that the formation of cyanate is a necessary intermediate step in both synthesis and decomposition. But others assume that the reaction proceeds through the rearrangement of ammonium carbonate to ammonium carbamate with the loss of water, and then by the dehydration of the ammonium carbamate to urea.

The two views may be written schematically,



The evidence is contradictory.

Sumner, Hand, and Holloway<sup>14</sup> found that no cyanate was formed when urea was decomposed in the presence of a very pure crystallized urease. Mack and Villars<sup>15</sup> found cyanate among the decomposition products but concluded that only the reaction by way of carbamate is catalyzed by urease. Fearon<sup>16</sup> has recently confirmed Sumner's results, but he suggests that the cyanate path may be an alternative one and that possibly it is catalyzed by another enzyme present in crude preparations of urease.

For present purposes it is only necessary to assume that there is present in the sap an enzyme capable of facilitating the urea equilib

<sup>11</sup> Ammonium carbonate solution contains according to the pH, bicarbonate and carbamate ions also.

<sup>12</sup> For recent syntheses of urea see Mack, E., and Villars, D. G., *J. Am. Chem. Soc.* 1923, 45, 501. Kay, H. D., *Biochem. J.* London 1923 17, 277. Fearon, W. R., *Biochem. J.*, London, 1936, 30, 1652.

<sup>13</sup> Werner, E. A. *The chemistry of urea*. London, Longmans, Green and Co. 1923.

<sup>14</sup> Sumner, J. B., Hand, D. B., and Holloway, R. G., *J. Biol. Chem.*, 1931 91, 333.

<sup>15</sup> Mack, E., and Villars, D. G., *J. Am. Chem. Soc.* 1923, 45, 501.

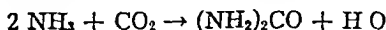
<sup>16</sup> Fearon, W. R., *Biochem. J.* London 1936, 30, 1652.

rium The path is not important It is a well established fact that many enzymes, including urease, operate most effectively at an optimum pH

In the urea-urease system, which unfortunately has been studied mostly with urea as the starting point, the equilibrium is set up most rapidly at pH 7.0 according to the results of Van Slyke and Zacharias<sup>17</sup> However, the addition of neutral salts or dilution of the urea solution causes the optimum pH to increase This shift has been confirmed by Lovgren<sup>18</sup> Recently, however, Howell and Sumner<sup>19</sup> have shown that though an optimum pH exists it depends not only on the concentration of urea but even more so on the type of buffer used in the system In phosphate buffers the optimum may be as high as 7.6, but in acetate the optimum in the same urea concentration is as low as 6.7 The authors show in the case of acetate buffers that the enzyme is still active at pH's as low as 3.0 and as high as 7.5 In phosphate buffers the range is from 5.0 to 9

If urea is concerned in the loss of ammonia from the sap it may perhaps operate in the following way The entering ammonia forms  $\text{NH}_4\text{X}$  at the outer surface and this diffuses into the sap where it is transformed to ammonium chloride We assume that  $\text{NH}_4\text{Cl}$  cannot diffuse out rapidly since we know from experiments that it cannot diffuse in rapidly<sup>20</sup> We suppose therefore that ammonia goes out chiefly as urea, the formation of which goes on in such fashion as to explain the induction period

The urea is formed from  $\text{CO}_2\text{-NH}_3$  species in the sap in the presence of an enzyme At the moment the cells are transferred from ammonia sea water to normal sea water the pH of the sap is higher than normal and nearer the optimum pH of the enzyme, consequently urea is formed rapidly and diffuses out of the sap<sup>21</sup> This is the equivalent of removing ammonium carbonate from the sap since



<sup>17</sup> Van Slyke, D. D., and Zacharias, G., *J. Biol. Chem.*, 1914, **19**, 181

<sup>18</sup> Lovgren, S., *Biochem. Z.*, Berlin, 1921, **119**, 215

<sup>19</sup> Howell, S. F., and Sumner, J. B., *J. Biol. Chem.*, 1934, **104**, 619

<sup>20</sup> Cooper, W. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, **14**, 117

<sup>21</sup> During the accumulation of ammonia we suppose that the loss of urea goes on, but in this case more ammonia is entering as  $\text{NH}_4\text{X}$  This is decomposed by carbonic acid and thus keeps the pH up

hence the pH is decreased. As pointed out previously only a very small amount of ammonia need be lost in this first process to lower the pH. The effect of lowering the pH is to make the enzyme less active so that a comparatively long time elapses before sufficient urea is formed to cause the ammonia concentration of the sap to decrease at a measurable rate. Hence an induction period is observed.

If we assume that the entrance of ammonia depends on the inward diffusion of  $\text{NH}_4\text{X}$ , and the exit of ammonia on the outward diffusion of urea, we run into a number of difficulties. If we call<sup>22</sup> the outer protoplasmic surface  $\lambda$  and the inner surface  $Y$  we may say that although the  $\lambda$  and  $Y$  layers of the protoplasm are undoubtedly different it is hard to see how a species which can diffuse in one direction in either layer cannot likewise diffuse in the opposite direction in the same layer. In particular  $\text{NH}_4\text{X}$ , if it enters the sap, must have diffused through  $Y$ . It ought then to be able to diffuse back through  $Y$  when the direction of the gradient is reversed by exposing the cells to running normal sea water. It is, of course, possible that no appreciable amount of  $\text{NH}_4\text{X}$  can form at the sap protoplasm interface because there is little or no  $\text{HX}$  there. This could happen if  $\text{HX}$  is not formed in  $Y$ . But in that case we should expect it to diffuse into  $Y$  and to the sap  $Y$  interface rather rapidly. Of course, if  $\text{HX}$  were formed only at the  $X$  sea water interface and if its partition coefficient were low it might be lost in great part to the sea water. In that case in order for there to be any appreciable diffusion of  $\text{NH}_4\text{X}$  through the protoplasm the partition coefficient of the salt  $\text{NH}_4\text{X}$  would have to be much greater than that of the acid  $\text{HX}$ . This does not seem probable. Indeed we have supposed that  $\text{HX}$ , which is probably not one acid but the type member of a group of weak acids, is much more soluble in the non aqueous protoplasmic surface than in aqueous solution.

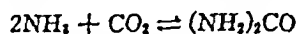
To avoid the assumption that a one way permeability exists in any layer it might be assumed<sup>23</sup> that  $\lambda$  is permeable to  $\text{NH}_4\text{X}$  but not to urea and  $Y$  is permeable to urea but not to  $\text{NH}_4\text{X}$ . On this basis the

<sup>22</sup> Osterhout W J V *Ergebn Physiol*, 1933, 35, 967

<sup>23</sup> Electrical evidence indicates that  $\lambda$  and  $Y$  are quite different (cf foot note 22)

seat of formation and decomposition of both urea and  $\text{NH}_4\text{X}$  might be in the  $W$  layer, the aqueous layer in the protoplasm between the two non-aqueous layers  $X$  and  $Y$ . Let us suppose that urea is formed or decomposed at the sap- $Y$  and  $Y$ - $W$  interfaces, and  $\text{NH}_4\text{X}$  is formed or decomposed at the sea water- $X$  and  $X$ - $W$  interfaces. Under these conditions, when the cell is exposed to ammonia containing sea water,  $\text{NH}_4\text{X}$  is formed at the sea water- $X$  interface and diffuses to  $W$ , where it is decomposed by  $\text{CO}_2$  which is either formed at the  $W$ - $Y$  interface or is diffusing from the sap- $Y$  interface. If  $\text{NH}_4\text{X}$  cannot diffuse in  $Y$  it might accumulate as  $\text{NH}_4\text{HCO}_3$  in  $W$  until  $\text{NH}_{3s} = \text{NH}_{3w}$ , but its entrance raises the pH of  $W$  and as a result the rate of formation of urea is increased as the enzyme activity increases. The urea diffuses through  $Y$  to the sap, but it cannot, we assume, diffuse much in  $X$  because of the low partition coefficient. The first urea entering the sap and decomposing there increases the concentration of undissociated ammonia and the pH is raised. This promotes the outward movement of  $\text{KX}$  for which there is a favorable partition coefficient in both  $Y$  and  $X$ . As a result the further decomposition of urea in the sap does not raise the pH since the loss of  $\text{KX}$  is equivalent to the loss<sup>4</sup> of  $\text{KOH}$ .

One point remains to be cleared up. Since the pH of the sap is low when the urea first enters its rate of decomposition would be small so that we might expect an induction period in the entrance of ammonia. But we have never observed one. Two things contribute to make it unlikely that we should see one. First the amount of ammonia required to raise the pH is very small because of the poor buffer capacity of the sap, and second the reaction



is in equilibrium far over on the side of the reactants. Consequently even though the efficiency of the enzyme is low, it takes a comparatively short time to produce an increase in the pH of sap. Once this occurs the efficiency of the enzyme increases.

Let us now consider conditions at the steady state. We should expect the entrance of ammonia to cease as soon as concentrations of urea in sap and  $W$  are equal, which should be when the condition  $(\text{NH}_3)_w^2(\text{CO}_2)_w = (\text{NH}_3)_{sap}^2(\text{CO}_2)_{sap}$  is fulfilled. But this cannot

happen as long as  $\text{NH}_4\text{X}$  is entering  $W$  and is being decomposed by  $\text{CO}_2$  to form  $\text{NH}_4\text{HCO}_3$ . This process, however, will cease when

$$(\text{NH}_4)_{e,w} = (\text{HX})_{p,e} = (\text{NH}_4)_w(\text{HX})_{e,p}$$

where  $e,p,w$  refers to the equilibrium layer in the  $X$  layer adjacent to  $W$ . But if  $(\text{HX})_{e,p} = (\text{HX})_{e,w}$  which will be the case if  $\text{HX}$  is distributed equally throughout the  $X$  layer, the right hand terms in numerator and denominator cancel, and then at the steady state

$$(\text{NH}_4)_{e,w} = (\text{NH}_4)_w$$

and a steady state throughout the system will occur when

$$(\text{NH}_4)_{e,w}(\text{CO}_2)_w = (\text{NH}_4)_{s,p}(\text{CO}_2)_{s,p}$$

But if  $(\text{CO}_2)_{s,p} = (\text{CO}_2)_w$ , which may well be the case, the steady state will occur when

$$(\text{NH}_4)_{e,w} = (\text{NH}_4)_{s,p}$$

or when

$$(\text{NH}_4)_{e,w} = (\text{NH}_4)_{s,p}$$

But from a previous paper<sup>3</sup> we derive the relationship that

$$(\text{NH}_4)_p(\text{HX})_p = (\text{NH}_4)_p(\text{HX})_e$$

at the steady state. In this expression  $e,p$  and  $e,w$  refer merely to particular layers of sea water and sap adjacent to the protoplasm, and for purposes of comparison of this expression with the one where  $e,p$  and  $e,w$  can be considered respectively equivalent to sea water and sap respectively. Further we can assume that corresponding activity coefficients in sap and sea water are equal and their concentrations may be substituted for activities.

Using data from the experiment described in the previous paper<sup>3</sup> we find that the steady state becomes  $= 0$  when  $[\text{HX}]_p - [\text{HX}]_{e,p}$  is taken as equal to 6.7.

Now suppose we identify  $\text{HX}$  with  $\text{CO}_2$ . In order to satisfy the urea steady state equation  $(\text{CO}_2)_p - (\text{CO}_2)_w$  will have to be equal<sup>24</sup>

<sup>24</sup> Because for the steady state when urea is concerned

$$\frac{[\text{NH}_4]_w}{[\text{NH}_4]_{s,p}} = \frac{[\text{CO}_2]_{s,p}}{[\text{CO}_2]_{e,w}} \quad \begin{aligned} [\text{NH}_4]_{e,w} &= 5.9 \times 10^{-4} \\ [\text{NH}_4]_{s,p} &= 8.83 \times 10^{-4} \end{aligned}$$

to 45 This seems very unlikely in the case of  $\text{CO}_2$  which, as we know from previous experiments,<sup>25</sup> diffuses very rapidly in the protoplasm For this reason it seems unlikely that urea plays any part in the process of ammonia penetration if we regard  $\text{HX}$  as  $\text{CO}_2$

Urea has served merely as an example of a possible species which might operate through an enzyme Instead of forming urea with  $\text{CO}_2$  ammonia might add on to an unsaturated linkage of an organic molecule elaborated by the protoplasm And if one molecule of ammonia adds one molecule of the organic compound, we get for the two equations

$$(\text{NH}_3)_v(R)_w = (\text{NH}_3)_s(R)_w$$

and

$$\frac{(\text{NH}_3)_s \cdot (HX)_s \cdot w}{(\text{NH}_3)_w (HX)_s \cdot w} = (\text{NH}_3)_v (HX)_s \cdot w$$

where  $R$  is an unsaturated molecule capable of adding on one amino group If  $\text{HX}$  is distributed uniformly in the  $X$  layer of the protoplasm, we may combine the two equations to get

$$(\text{NH}_3)_s \cdot w(R)_w = (\text{NH}_3)_{\text{esp}}(R)_{\text{esp}}$$

which is the same sort of equation as we obtained in the previous paper

Before discussing possible reactions of the type suggested we shall consider the effects of light on the urea equilibrium

It was observed that normal light increased both the rate of accumulation and the rate of exit of ammonia and that the induction period was shorter under normal light

The increased rate of entrance in light is readily explained by assuming that due to photosynthesis the pH immediately at the sea water- $X$  interface is raised so that the rate of diffusion of  $\text{NH}_4\text{X}$  through  $X$  is increased This increases the rate of urea formation and therefore the rate of increase of the ammonia concentration in the sap But the slowing up of the rates of exit and the lengthening of the induction period in the dark do not fit as well into the picture

The ammonia emerging from the protoplasm is washed away by the flow of sea water to which the cells are exposed Nevertheless there is a small but definite concentration of undissociated ammonia

<sup>25</sup> Jacques, A G, and Osterhout, W J V, *J Gen Physiol*, 1929-30, 13, 695

in the sea water and this should increase in the light at the sea water protoplasm interface, so that the rate of diffusion of  $\text{NH}_4\text{X}$  through  $\lambda$  should be decreased. The effect might be very small indeed because of the very low concentration of total ammonia in the sea water, but any effect would be in the opposite direction to that observed.

Light, however, may affect the rate of attainment of the urea equilibrium in other ways. It is not impossible that light alone affects the rate of urea synthesis and decomposition<sup>26</sup>. But it seems more likely that the results may arise from an increase in the activity of the enzyme.

Some recent results by Murakami<sup>27</sup> indicate that a number of enzymes, among them urease, are quite inactive in the dark, but become active when illuminated. However, the activity decreases as the intensity of the light increases. But Pincussen and Katô<sup>28</sup> found that prolonged exposure to ultraviolet light or sunlight gradually inactivates urease. Assuming, however, that moderate light increases the effectiveness of the enzyme in the cell we can see that this would explain all the observed effects. Especially it would explain why in the exit experiments the induction period is shorter in the light. For if the enzyme is more effective the rate of increase in the urea concentration in the sap will be greater, and the urea concentration at which the decrease of ammonia concentration in the sap begins to be appreciable will be attained sooner. Light might also influence the rate by increasing the amount of enzyme in the system. As is well known in general the rate at which equilibrium is attained in enzyme catalysis increases with the increase in the ratio of enzyme to substrate.

The question may be asked if it is necessary to assume a dual process for ammonia transport using both  $\text{NH}_4\text{X}$  and urea. There is the possibility that urea alone accounts for ammonia entrance and exit.

<sup>6</sup> For photolysis of urea in sunlight see Rao G G, and Pandala, K M, *J Indian Chem Soc*, 1934 11, 623. For the synthesis of urea from ammonium carbonate solution in ultraviolet light see Fearon, W R, and M'Kenna, C B *Biochem J* London 1927, 21, 1087.

<sup>7</sup> Murakami R *J Agric Chem Soc, Japan* 1936 12, 151, 172, 180 709 1937 13, 46. These papers are in Japanese but English abstracts are supplied.

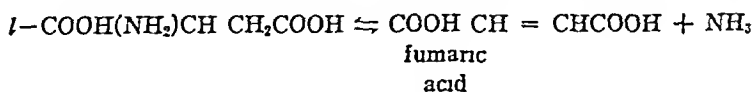
<sup>28</sup> Pincussen L and Katô N, *Biochem Z*, Berlin, 1923 142, 228.



On this basis urea must be formed during entrance at the protoplasm-sea water interface in the presence of an enzyme. In the light several effects might be present (a) the light might increase the effectiveness of the enzyme thereby speeding up the entrance of ammonia, (b) the light by inducing photosynthesis would raise the pH at the sea water-protoplasm interface and this might speed up entrance by bringing the system nearer the optimum pH for the enzyme, (c) the light might slow up the reaction by causing the photosynthetic removal of  $\text{CO}_2$  and thus decreasing the concentration of one of the reactants needed for urea synthesis, or (d) the light might adjust the pH at a less favorable point for the enzyme, should the normal pH of sea water be nearer the optimum pH than that produced by photosynthesis. Thus light might have the net effect of increasing the speed greatly, leaving the speed unchanged, or decreasing it to greater or less extent.

If it is to increase the speed we should have to suppose that the enzyme effectiveness increases all the way from the pH of the sap about 5.6 up to 9.0-9.5 the probable pH in the layer of sea water adjacent to the protoplasm. This would be an unusually long range.

We now consider possible carriers other than urea, such as the amino acids, aspartic acid,  $\text{COOH}(\text{NH}_2)\text{CH}_2\text{COOH}$ , asparagine,  $\text{COOH}(\text{NH}_2)\text{CH}_2\text{CONH}_2$ , and glutamine,  $\text{COOH}(\text{NH}_2)\text{CH}_2\text{CH}_2\text{CONH}_2$ . All these are widely distributed in plants, particularly the last two which usually occur together.<sup>29</sup> All of these can lose ammonia in the presence of suitable enzymes. Aspartic acid can be deaminized readily in the presence of aspartase to fumaric acid. Thus,



This reaction, according to Jacobsohn and Tapadinhas<sup>30</sup> is a true reversible catalysis. The equilibrium is established according to results of Quastel and Woolf,<sup>31</sup> and Woolf<sup>32</sup> who used an enzyme preparation extracted from micro-organisms when  $K \frac{[\text{NH}_3][\text{fumarate}]}{[l\text{-aspartate}]} = 0.01$ . Borsook and Huffman<sup>33</sup> have

<sup>29</sup> Schwab, G, *Planta*, 1936, 25, 579

<sup>30</sup> Jacobsohn, K. P., and Tapadinhas, J, *Biochem Z*, Berlin, 1935, 282, 374

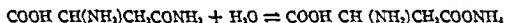
<sup>31</sup> Quastel, J. H., and Woolf, B, *Biochem J*, London, 1926, 20, 545

<sup>32</sup> Woolf, B, *Biochem J*, London, 1929, 23, 472

<sup>33</sup> Borsook, H., and Huffman, H. M., *J Biol Chem*, 1932, 99, 663

calculated the free energy changes in the production of fumaric acid from *l* aspartic acid, and have compared the result with that calculated from the equilibrium given by Quastel and Woolf. They agree with Jacobsohn and Tapadinhas that the reaction is a real reversible catalysis.

The optimum pH for the enzyme appears to be between 7.0 and 7.5,<sup>24</sup> and at 5.5 the action ceases. *l* asparagine can be hydrolyzed in the presence of a specific enzyme, asparaginase, to *l* aspartic acid, but it is not certain that this is a really reversible catalysis. Some investigators believe that the asparagine is completely hydrolyzed in the presence of the enzyme. Geddes and Hunter<sup>25</sup> support this view. They fix the optimum of the reaction about at pH 7.9 and find that it is still active down to 5.5 and up to 10.5. Suzuki<sup>26</sup> also found that the asparagine was completely hydrolyzed. His optimum value was 8.10. However, the results of Bach<sup>27</sup> who was unable to get more than 80 per cent hydrolysis, suggest that an equilibrium is set up. Bach's optimum pH was 8.6 and the range of enzyme activity is from pH 6.5 to pH 10. Schmalfuss and Mothes<sup>28</sup> also believe that the reaction is a reversible one resulting in the formation of a salt, ammonium aspartate,



They fix the optimum at 7.7 – 7.8 and the range of activity of the enzyme from pH 6 to pH 10. It seems probable to us that the reaction is a reversible catalysis, but that the equilibrium point is far over on the side of the aspartic acid.

Whether or not this is so, it is clear that in the plant asparagine can be formed, possibly through the effect of another enzyme.<sup>29</sup>

<sup>24</sup> According to Virtanen and Tarnanen (Virtanen, A. I., and Tarnanen, J., *Biochem. Z.*, Berlin, 1932, 260, 193) the optimum is from 7.0 to 7.5 and the enzyme is no longer effective at 5.5.

<sup>25</sup> Geddes, W. F. and Hunter, A., *J. Biol. Chem.*, 1928, 77, 197.

<sup>26</sup> Suzuki, Y., *J. Biochem.*, Japan, 1936, 23, 57.

<sup>27</sup> Bach, D., *Bull. Soc. chim. biol.*, 1929, 11, 119.

<sup>28</sup> Schmalfuss, K., and Mothes, K., *Biochem. Z.*, Berlin, 1930, 221, 134.

<sup>29</sup> In this connection, Kultzscher (Kultzscher, M., *Pflanz.*, 1932, 17, 699) says that the equilibrium, amide nitrogen  $\rightleftharpoons$  ammonium salts, varies greatly with the pH of the sap. In plants with saps below pH 5 the tendency is to store nitrogen as ammonium salts and above that as amide. This suggests to us that in plants with saps of low pH the enzyme is so ineffective that amides such as asparagine and glutamine cannot form very rapidly. It should be noted that the  $\text{NH}_2$  groups in aspartic and glutamic acids are amino groups, not amide groups. An amino group is not hydrolyzed off in the presence of a hydrolase such as asparaginase. Indeed, apparently it cannot be removed by hydrolysis which would leave an hydroxy acid, under natural conditions. But instead the amino group is oxidized off as ammonia, leaving behind an unsaturated acid.

The situation with glutamine is not yet clear. The recent work of Krebs<sup>40</sup> indicates that there are at least two enzymes which can be classed as glutamases. The one obtained from kidney tissue appears capable of hydrolyzing glutamine or causing its synthesis from glutamic acid and an ammonium salt. The optimum pH is at 7.4 and the range from 5.9 to 8.6 at least. The author did not explore the limits further.

Now if any one of these amino acids acts as ammonia carrier it may be expected to operate in the following way. If aspartic acid is concerned. In the presence of the enzyme fumaric acid acquires an amino group by uniting with undissociated ammonia thus setting up a gradient of aspartic acid across the protoplasm. The aspartic acid moves to the protoplasm-sap interface where it is decomposed in the presence of the enzyme and the undissociated ammonia goes into the sap. If this is the correct explanation accumulation could occur until  $[\text{NH}_3]_i = [\text{NH}_3]_o$ , provided the fumaric acid is distributed uniformly through the protoplasm if equilibrium could ever be established. In most cases we should not expect this. Instead, owing to the lower pH at the sap-protoplasm interface the enzyme would be less effective. Hence the concentration of aspartic acid at the sap-protoplasm interface would equal that at the sea water-protoplasm interface before  $[\text{NH}_3]_i = [\text{NH}_3]_o$ .

Either asparagine or glutamine would operate in the same way as aspartic acid except that the reaction in this case involves the loss of a molecule of water per molecule of ammonia reacting.

It is unnecessary to carry the argument further. The same considerations applying to the transport of ammonia as urea could apply to its transport as an amino group. But if ammonia enters by the formation of an amino group the scheme which best fits the experimental facts is as follows.

All the ammonia is transported as amino groups which add on to the unsaturated linkage of an organic species  $R$  elaborated wholly or chiefly at the sap-protoplasm interface.

This is preferable because the rate equation during accumulation which can be derived from it is analogous to the one found to fit in the previous paper except that in place of the acid  $\text{HX}$  we substitute the organic species  $R$ .

In a previous paper<sup>3</sup> the rate equation could be put in the form

$$P''' = \frac{2.3}{t} \log \frac{\frac{b}{c}a}{\frac{b}{c}a - x}$$

<sup>40</sup> Krebs, H. A., *Biochem. J.*, London, 1935, 29, 1951

where  $P'''$  is the permeability constant,  $b$  = concentration of HX or alternately of  $R$  at the sea water protoplasm interface,  $c$  = concentration of HX or  $R$  at the sap protoplasm interface, and  $a$  and  $x$  are the concentration of undissociated ammonia in sea water and sap respectively. In the previous paper we interpreted the results to mean that

$$[H\lambda]_{pi} = 6.7[Hx]_{po}$$

In the present case we assume that the effective concentration of  $R_{spi} = R_{spo}$ . The necessity for introducing  $R$  in place of  $H\lambda$  is apparent only when we consider the exit of ammonia. The neutralization reaction to form  $NH_4\lambda$  must be regarded as practically instantaneous, but the amination of the  $R$  may be slow and the induction period can be attributed to the relative ineffectiveness of the enzyme as a catalyst under certain conditions.

#### SUMMARY

The exit of accumulated ammonia from the sap of *Valonia macrophylla*, Kütz., into normal (nearly ammonia free) sea water, has been studied in light (alternation of daylight and darkness) and in darkness. Exit is always preceded by an induction period lasting 1 or more days. This is longer in darkness. After exit starts the rate is greater in light than in darkness.

The pH of the sap drops off soon after the cells are exposed to normal sea water even before any definite decrease in the ammonia concentration of the sap has occurred. This suggests that the decrease in the pH is due to the loss of a very small amount of  $NH_3$  or  $NH_4OH$  without a corresponding gain of sodium as a base.

In most cases sodium replaced the ammonia lost during exit, but there is some evidence that potassium may also replace ammonia.

To account for the induction period it is suggested that other species than  $NH_4Y$  are concerned in the transport of ammonia, for example urea or amino acids.



# THE ELECTROPHORETIC MOBILITY OF RABBIT ERYTHROCYTES AND GHOSTS

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(Accepted for publication, December 5, 1938)

Between 1929 and 1934 Abramson (1929, 1934) carried out many measurements of the electrical mobilities of red cells and of the ghosts produced by various forms of hemolysis. He found that after hemolysis by water, the mobility is unchanged, as it also is if lysis is produced by small quantities of saponin, or by small amounts of complement, he therefore concluded that hemolysis can occur without necessitating a radical change in the chemical constitution of the surface, and put forward the idea that for hemolysis to occur it may be necessary to affect only certain "key spots," occupying only a very small portion of the cell surface.

The purpose of this paper is to extend the observations, and to bring the results into relation with those obtained by certain other methods.

## *Methods and Preparations*

The electrophoresis measurements were made in the Abramson horizontal micro-electrophoresis cell, and the technique used was that developed by Abramson (1929-1934) and more recently described by Moyer (1936). The electrophoretic mobilities were obtained from measurements at the stationary levels as checks in the majority of determinations; however, measurements were also made at levels throughout the cell and parabolas plotted. The mobilities obtained by the integration of these parabolas always agreed within 5 per cent with the corresponding mobilities obtained from stationary level measurements. The measurements were all carried out at room temperature and the mobilities converted to mobilities at 25 C by allowing a 2 per cent increase per degree. Although the same electrophoresis cell was used for most of the measurements a few of the determinations on unlysed red blood cells and watery ghosts were made in a second cell of the same type. The mobilities obtained in the two different cells checked exceedingly well with one another.

The solution in which the red blood cells and ghosts were suspended for

electrophoretic measurements was an isotonic buffer-glucose solution of pH 7.2. It was made by mixing 10 cc of a M/15 phosphate solution (prepared by adding 70 cc of M/15  $\text{Na}_2\text{HPO}_4$  to 28 cc of M/15  $\text{KH}_2\text{PO}_4$ ) and 90 cc of a 5.4 per cent glucose solution. The buffer-glucose solutions were all freshly prepared. In some determinations, as will be pointed out later, various concentrations of saponin were also included in the phosphate buffer-glucose solution. The unlysed erythrocytes, ghosts, oil droplets, and quartz particles on which mobility determinations were made were always first suspended in suitable concentration in 1 per cent NaCl before being added to the buffer-glucose solutions, 0.3 cc of saline suspension being mixed with 40 cc of buffer-glucose solution. The specific resistance of each suspension was determined at the same temperature at which the measurements were made.

The blood from which the cells were obtained was fresh, defibrinated rabbit blood. The cells were first washed three times with 1 per cent saline and finally suspended in 1 per cent saline.

"Watery ghosts," formed by lysis of erythrocytes with a hypotonic solution, were prepared as follows. The cells from 10 cc of defibrinated blood, after three washings, were made up to the original volume by the addition of 1 per cent saline, and to the 10 cc of cell suspension 60 cc of water was added. After about 10 minutes, 10 cc of 7 per cent saline was added, making the resulting 80 cc of suspension fluid isotonic. The suspension was immediately centrifuged at high speed in celluloid cups for 15 minutes, the supernatant fluid removed, and the ghosts suspended in saline. No attempt was made to free them of the hemoglobin remaining in them.

"Freezing-and-thawing ghosts" were prepared as follows. A suspension of cells from 20 cc of defibrinated blood, twice washed, was placed in the bottom of a 250 cc beaker, and the beaker set upon a flat cake of dry ice. The suspension was allowed to freeze solidly, and then to thaw at room temperature. The resulting hemolyzed cells were then washed from one to three times, the number of washings being sometimes limited by the low yield of ghosts, and were finally suspended in 1 per cent saline.

"Chloroform ghosts," the result of hemolysis with chloroform, were prepared as follows. A suspension of red cells from 10 cc of defibrinated blood, twice washed, was added to about 150 cc of 1 per cent saline saturated with chloroform. After about 10 minutes had been allowed for hemolysis, the suspension was subjected to high speed centrifugation for about 15 minutes. The ghosts thrown down were washed and finally suspended in 1 per cent saline in the same manner as the freezing-and-thawing ghosts. Both freezing-and-thawing ghosts and chloroform ghosts, if washed three times, are quite white in color.

"Saponin ghosts" were made as follows. Washed cells were added to the already described phosphate buffer-glucose solution (0.3 cc of cell suspension to 40 cc of solution), containing in this case, however, also enough saponin (0.1 per cent or 0.01 per cent) to hemolyze them. Electrophoresis measurements were made directly on the saponin ghosts in the solutions in which they were prepared. In connection with saponin ghosts, mobility measurements were also made on

unlysed red blood cells in buffer glucose solutions containing insufficient concentrations of saponin for lysis, namely 0.001, 0.0001, and 0.00001 per cent of saponin.

Mobility measurements were also made on microscopic quartz particles and paraffin oil droplets in buffer glucose solutions containing various concentrations of saponin. The suspension of oil droplets was made by shaking the oil vigorously with 1 per cent saline for about half an hour. The droplets of which the mobilities were determined were about  $1\ \mu$  in diameter.

### RESULTS

In Table I are given the mobilities found for unhemolyzed rabbit erythrocytes and for ghosts of rabbit erythrocytes prepared by lysing

TABLE I

Condition of cells	Mobility	Average mobility
	$\mu/\text{sec}/\text{volt}/\text{cm}$	$\mu/\text{sec}/\text{volt}/\text{cm}$
Unlysed cells	1.01 (a)	1.03
	1.03 (b)	
	1.02 (c)	
	1.01 (d)	
	1.03	
Watery ghosts	1.11	1.03
	1.00	
	1.01 (a)	
	1.02 (b)	
Freezing and thawing ghosts	1.08	1.05
	1.02 (d)	
Chloroform ghosts	1.15	1.03
	1.00 (c)	

with water, by freezing and thawing, and with chloroform. The mobilities of all are within experimental error of one another. In several cases both the mobility of the unlysed cells and that of the ghosts in question was determined in the same suspension in the electrophoresis cell at the same time. The excellent agreement of such simultaneously determined mobilities, which are marked by similar letters in the second column of Table I, shows that there is no detectable difference between the mobility of unhemolyzed rabbit erythrocytes and the mobilities of their ghosts formed by lysing with water, by freezing and thawing, or with chloroform. Further evidence of the



agreement of these mobilities is presented in Fig 1 In it electrophoretic mobilities of intact red cells, watery ghosts, freezing-and-thawing ghosts, and chloroform ghosts at various depths in the same electrophoresis cell (in separate suspensions in separate determinations, however) are plotted against the fraction of depth in the cell

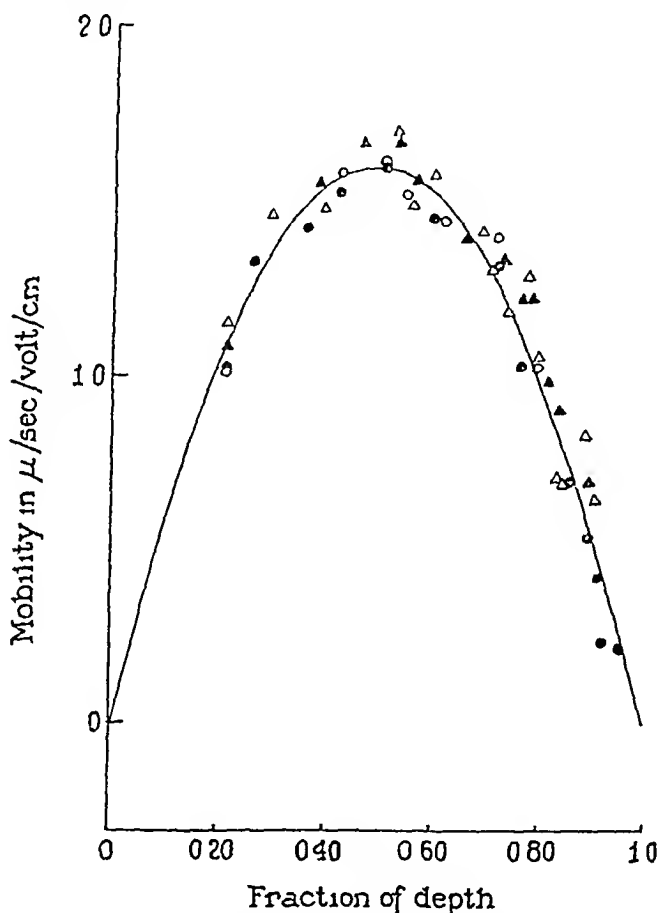


FIG 1 Closed circle, unlysed cells, open circle, watery ghosts, closed triangle, freezing-and-thawing ghosts, open triangle, chloroform ghosts

Theoretical parabola obtained from plotted points by method of least squares

In Table II are presented the mobilities of unhemolyzed rabbit erythrocytes and saponin ghosts in phosphate buffer-glucose solutions containing various concentrations of saponin As mentioned above, unlysed red cells were added directly to these solutions, and in the two solutions containing the higher concentrations of saponin the cells

were hemolyzed. The mobilities in Table II were determined from a series of measurements made in a single day, and all of the mobilities agree with themselves and with the mobilities in Table I within experimental error. The unlysed rabbit red cells in the presence of various non lytic concentrations of saponin, and the saponin ghosts in the presence of lytic concentrations, have the same electrophoretic mobility, as far as our experimental results indicate, as unlysed rabbit red cells, watery ghosts, freezing and thawing ghosts, and chloroform ghosts in saponin free solutions.

TABLE II

Saponin concentration	Mobility	Condition of cells
<i>per cent</i>	<i><math>\mu</math>/sec /volt/cm</i>	
Zero	1.08	Unlysed
0.00001	1.09	"
0.0001	1.09	"
0.001	1.17	"
0.01	1.16	Ghosts
0.1	1.09	"

TABLE III

Saponin concentration	Quartz mobility	Oil mobility
<i>per cent</i>	<i><math>\mu</math>/sec /volt/cm</i>	<i><math>\mu</math>/sec /volt/cm</i>
Zero	3.47	3.81
0.0001	3.47	—
0.001	3.40	3.48
0.01	3.19	2.98
0.1	2.69	2.75

In view of the possibility that saponin might form an adsorbed film on the red cell surface, an attempt was made to determine the mobility of saponin covered surfaces. Table III gives the mobilities of quartz particles and paraffin oil droplets in phosphate buffer dextrose solutions containing various concentrations of saponin. The mobilities for quartz particles and oil droplets at the same intermediate saponin concentrations are not strictly comparable, for the quartz particles were in very much greater concentration (thus affording more surface) in suspension than were the oil droplets. However, in the suspensions

containing 0.1 per cent saponin, where there was surely enough saponin to cover all of the surface of the quartz particles and oil droplets, the mobilities are within experimental error of each other. It would appear from these results that saponin is adsorbed on both quartz particle and oil droplet, lowering their mobilities with increasing adsorption until they both have complete surface films of saponin and therefore the same mobilities. The electrophoretic mobility of saponin-covered surfaces therefore seems to be somewhat over  $2.5 \mu/\text{sec}/\text{volt}/\text{cm}$ , considerably higher than the mobility of about  $1.07 \mu/\text{sec}/\text{volt}/\text{cm}$  of unlysed red cells and saponin ghosts in the same buffer-glucose solution containing various concentrations of saponin.

### DISCUSSION

It is surprising that these different forms of lysis, which correspond to a variety of degrees of injury to the red cell, should be unaccompanied by any change in  $\zeta$ -potential. The watery ghosts have a resistance and capacity virtually the same as that of the intact cell (Fricke and Curtis, 1935), although, over a period of minutes, they are permeable to cations (Davson and Ponder, 1938), on the other hand, they are impermeable to hemoglobin (Fricke, Parker, and Ponder, 1939), and exhibit the typical biconcave shape of the mammalian red cell. So far as it goes, the evidence is that this form of lysis is the result of the cell membrane being stretched beyond its normal area (Ponder, 1937, Castle and Daland, 1937), and that the permeability to pigment which results is followed by some sort of repair process. The resistance and capacity of the freezing-and-thawing ghosts is also like that of the intact cell, although there is a peculiar frequency dependence of the capacity (Fricke and Curtis, 1935), these ghosts are also slowly permeable to cations. But they are permeable to hemoglobin,<sup>1</sup> and the biconcave shape is lost. The action of the chloroform is presumably to dissolve out or otherwise disorient the lipid components of the cell ultrastructure, the chloroform ghosts are permeable to cations and to pigment, and again the biconcave shape is lost. The

<sup>1</sup> At least in the direction from inside to outside, for they can be washed free of the residual hemoglobin which remains in watery ghosts. Watery ghosts are impermeable to hemoglobin placed in the fluid surrounding them, but we have no information about a similar impermeability of freezing-and-thawing ghosts.

action of saponin is probably the most drastic of all, for this lysis can react with both the protein and the lipid components of the membrane ultrastructure (*cf* Schulman and Rideal, 1937). The ghosts are roughly spherical, and are permeable to both cations and to pigment, and if sufficient saponin is used the resistance and capacity disappear entirely. Whether this is due to all the cells becoming more and more permeable as time goes on or as the concentration of saponin is increased, or to an increasing number of the cells becoming totally permeable in an all or none fashion, is not entirely clear. At all events, increasing quantities of the lysis are taken up by the cells as time goes on (Ponder, 1935), and this presumably corresponds to increasing injury.

Nevertheless the electrical mobility is the same in all cases, and the same as for the intact rabbit red cell. It is difficult to account for this, but there are at least two lines of speculation open.

The first of these is Abramson's key spot hypothesis, which suggests that lysis results from certain key spots in the cell membrane breaking down, the greater part of the surface, however, remaining much as it was before. The results of "fading time" experiments tend to support this view in the case of lysis by hypotonic solutions (Davson and Ponder, 1938), Ca stearate monolayers (Ponder and Neurath, 1938), dilute saponin solutions (Ponder and Marsland, 1935), and possibly chloroform (Davson and Ponder, 1938), the fading times are such as would be expected if there were 10 to 50 holes of 250 Å through which the pigment could escape, and their total area would be anything from 1/10,000th to 1/2,000th of the area of the cell surface.<sup>3</sup> The difficulty arises when we have to consider the case of concentrated

- Fricke and Curtis discuss the matter from the latter point of view. If the resistance and capacity of any cell are lost in an all or none fashion after saponin hemolysis and if the loss is followed by disintegration (as Fricke and Curtis seem to think), it may be that the ghosts whose electrical mobility we have measured are structures which still retain their normal resistance and capacity. The alternative possibility, that a partial or complete loss of resistance and capacity is accompanied by no change in mobility, would be very interesting.

<sup>3</sup> In order to account for many immunological reactions it is convenient to postulate the existence on the cell surface of key spots with specific reactivities but the key spots referred to in this paper are not necessarily of this kind. In the meantime, it is better to regard them as merely the weakest spots in a membrane which, from its very nature, is unlikely to be altogether homogeneous.

saponin solutions (*e g* 1 in 1000), for, whether as judged by fading times or in any other way, such concentrated solutions, acting over considerable periods of time, are unlikely to have so restricted an action

The second possibility is suggested by the particular form of the membrane ultrastructure (Schmitt, Bear, and Ponder, 1937 and 1938) As we now conceive it to be, the cell membrane has an outer, tangentially arranged, network or lattice of protein, probably the stromatin of Jorpes (1932) and Boehm (1935), and probably only a few molecules thick Within this, there are radially arranged layers of lipid, not more than two or four in number, and within this again is another layer of protein intimately connected with the gel of stromatin which Boehm believes, probably correctly, to fill the cell interior It is possible that it is the outer protein layer, with its polar groups, which is responsible for the electrical mobility, while lysis results from the destruction or disorientation of the more deeply seated non-polar lipid layers, which contribute little to the surface charge All the lytic agents considered, in fact, may leave the layer responsible for the electrical mobility relatively untouched <sup>4</sup>

The other point which emerges from this investigation is that the red cell ghost apparently does not adsorb a layer of saponin as do oil droplets and quartz particles This is of interest in view of the fact that the red cell surface is the only surface hitherto investigated which does not adsorb gelatin, apparently it has quite special properties

#### SUMMARY

Measurements of the electrical mobility of washed rabbit red cells and of ghosts produced by hypotonic solutions, freezing-and-thawing, chloroform, and saponin were made in the Abramson horizontal micro-electrophoresis cell These different forms of lysis, which corresponds to a variety of degrees of injury to the red cell, are unaccompanied by any change in electrical mobility These observations are discussed from the standpoint of the possible structure of the cell membrane and the action of lysins upon it

<sup>4</sup> The investigations of Monaghan and White (1936), and of Byler and Rozen-daal (1938) show that the reactivity of the cell surface to gelatin and chicken serum is somewhat altered These observers agree, however, that the electrical mobility of the hemolyzed watery ghost is the same as that of the normal cell

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# THE FLICKER RESPONSE CONTOUR FOR THE GECKO (ROD RETINA)

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(Accepted for publication, November 21, 1938)

## I

By cytological criteria the retina of the gecko is devoid of cones. The visual cells are structurally rods,<sup>1</sup> and are associated with a visual purple.<sup>2</sup> Determinations were made of the interdependence of flash frequency and flash illumination for threshold response to visual flicker, using the species *Sphaerodactylus inaguae* Noble. These measurements give a flicker response contour for flash cycles with equal light and dark times, at a particular temperature. The comparison of this function with that obtained for the turtle *Pseudemys*,<sup>3</sup> with pure cone retina, provides material for critically evaluating certain aspects of the duplexity doctrine of visual performance in vertebrates.

The duplexity doctrine<sup>4</sup> has endeavored to bring together the proof of the composite character of the curves of visual performance as seen in most vertebrates, the known presence in these animals of at least two chief histological types of receptor elements, the relations of these to the ethological types of predominantly diurnal or nocturnal activity, and the spatial distribution of excitability in the (human) retina. Retinal rods are held to be connected with scotopic, indistinct, colorless vision, cones with photopic vision, distinctness, and

<sup>1</sup> Rochon Duvigneaud A, *Ann d'oculist*, November, 1917, 16. Detwiler, S R, *J Comp Neurol*, 1923, 36, 125. Verner M L, *Bull biol France et Belgique*, suppl 20, 1935, 140 pp. 1937, 71, 238.

<sup>2</sup> Detwiler, S R, *J Comp Neurol*, 1923, 36, 125.

<sup>3</sup> *Proc Nat Acad Sc*, 1938, 24, 125, 216. *J Gen Physiol*, 1938-39, 22, 311.

<sup>4</sup> Cf citations in *Proc Nat Acad Sc*, 1938, 24, 125, 1939, 25, in press.

color The measurement of the flicker response contour affords a precise estimate of important features of visual performance, since it depends upon a limiting case of intensity discrimination <sup>5</sup> The data<sup>3</sup> on the turtle *Pseudemys*, with pure cone retina, gave an unequivocal instance of the direct correlation of one class of retinal elements with one class of excitation elements The  $F - \log I$  curve is here a simple probability integral over its whole extent, whereas for vertebrates having both rods and cones it is a duplex affair <sup>6</sup> This curve has the general properties ( $F_{max}$ , position on the intensity scale, and standard deviation of the first derivative) characteristic of the so called cone section of the duplex  $F - \log I$  curve for typical vertebrates It is of considerable interest to discover if a known purely rod retina will provide a similar comparison with the so called rod segment of the duplex curves

It could be expected that if this should be the case the  $F - \log I$  contour for the gecko would be a single probability integral, not duplex, rising to a comparatively low maximum, having a comparatively large  $\sigma'_{\log I}$  and a low value of the abscissa of inflection ( $\tau'$ ) In expecting a single simple curve we, of course, ignore possible effects associated with the occurrence of both single and double rods,<sup>2</sup> since in *Pseudemys*<sup>3</sup> no complications are introduced by the existence of double cones <sup>7</sup> The maximum  $F$  expected (under the same conditions of experimentation) could well be higher than for the "rod" segment in duplex curves, this could be due simply to the presence of a relatively larger number of excitation elements Conditions experimentally known to elevate  $F_{max}$  are increase of retinal area<sup>8</sup> and decrease of the light time fraction in the flash cycle,<sup>9</sup> these procedures also, and in the same proportion, lower  $\tau'$ , hence increase of  $F_{max}$  should in the gecko case be expected to tend to lower  $\tau'$ , and thus to

<sup>5</sup> *J Gen Physiol*, 1935-36, 19, 503, 1936-37, 20, 393, 1937-38, 21, 313

<sup>6</sup> *J Gen Physiol*, 1935-36, 19, 495, 1937-38, 21, 17, 203, etc

<sup>7</sup> That such effects are not impossible (although certainly not demonstrated) was suggested by the tripartite character of the  $F - \log I$  contour for the newt *Triturus* (*Proc Nat Acad Sc*, 1938, 24, 125 *J Exp Zool*, 1939, in press)

<sup>8</sup> *J Gen Physiol*, 1937-38, 21, 223

<sup>9</sup> *J Gen Physiol*, 1937-38, 21, 313, 463

assist rather than interfere in the analogy with rod properties in other vertebrates if the comparison is really appropriate

For reasons which may be relevant and partly valid in the long run, it was suggested<sup>10</sup> as a result of the analytical dissection of the duplex  $F - \log I$  contour for fishes that the rod contribution might be found to decline at higher intensities, if it could be separately examined in isolation. It naturally does not necessarily follow that (in terms of the duplicity theory) the flicker curve (whatever it may prove to be) for a completely color blind human being should exhibit this behavior, in the nature of the situation, one cannot very well demand to examine the retina in point under the microscope. From the standpoint of elementary logic a considerable fraction of the argumentation and interpretation involving the duplexity theory has been of an eminently circular character. Aside from all this, however, there is now a basis for the suggestion<sup>11</sup> that the decline of the rod curve may, in a duplex flicker contour, be the result of inhibitory influences due to the excitation of "cone" connected elements

## II

A supply of the small nocturnal gecko *Sphaerodactylus inaguae* Noble was collected for us at Matthew Town, Great Inagua, Bahama Islands, by Mr R A McLean in the summer of 1938. We are very grateful to him for this assistance, and for his bringing them to us. We are indebted to Dr Thomas Barbour, Director of the Museum of Comparative Zoology, for his identification of the species.

The animals were kept in insectary cages, provided with pieces of broken flower pots giving dark recesses and periodically supplied with living *Drosophila*. Over half the geckoes lived upwards of 3 weeks after arrival in Cambridge at room temperature 26–27.5°.

The apparatus used for tests involving response to visual flicker<sup>12</sup> is such as to require that the animals tested be rather small (not over about 12 cm long). The specimens of *Sphaerodactylus* were not over 7 cm in length. With a terrestrial reptile one cannot maintain the same control of temperature as is possible with the small aquatic forms we have used hitherto. The mean temperature was reasonably constant however, at 26.7°, with a P.E. of the dispersion of the readings on various days = 0.206°, the extreme deviations were at 26.4° and 27.1°.

<sup>10</sup> *J Gen Physiol*, 1936–37 20, 411 1937–38 21, 17

<sup>11</sup> *J Gen Physiol*, 1938–39 22, 463 *Proc Nat Acad Sc*, 1938, 24, 542

<sup>12</sup> *J Gen Physiol*, 1935–36 19, 495, 1936–37 20, 211

The temperature of lizards is likely to be a little below that of the environment<sup>13</sup> (due to evaporation of water), but probably by not more than 0.2°

*Sphaerodactylus* is not particularly active in the daytime. There appeared to be no definite indication of periodicity in this respect, day or night. Individuals were taken at random, a group of 10 being used for each test. On each of these three observations were taken. The average of the individual means ( $I_1$  or  $F_1$ ) is recorded as  $I_m$  or  $F_m$ .<sup>14</sup> The data are thus not homogeneous, since at the different points (Fig. 1) the individuals used are not the same group. The comparative general form of the  $F - \log I$  contour was the primary objective. Each set of readings was preceded by 45 minutes dark adaptation. When tested by exposure to gradually increased flash intensities, or to gradually reduced flash frequencies with fixed intensities,<sup>15</sup> the animal is usually motionless until at a certain level of  $I$  (or  $F$ ) there occurs a bending of the head against the direction of movement of the revolving stripes, with head nystagmus. This is the threshold response.<sup>16</sup> In some cases the gecko bends the head ventrally, and makes slight rotating motions with it. The critical readings were not based on this response, as it is difficult to see at low intensities. Head nystagmus occurs regardless of the position of the gecko in its jar, whether radially oriented with reference to the striped cylinder or parallel to the cylinder facing with or against the stripe motion. The response is inhibited by stereotropic effects if there is contact with the wall of the containing cylinder. The clearest threshold responses are obtained when the gecko is facing in the direction of rotation of the striped cylinder, but not parallel with the wall. The animals are so quiescent that they may be put in this position before observation is begun. Adaptation during the observations is not a factor, a series of as many as twelve readings taken in succession shows no trend in the critical intensity over some 10 minutes.

### III

Table I contains the results of determinations of critical flash frequency at fixed flash intensities, and of critical illumination at fixed flash frequencies. The flash cycle was of equally long light and dark intervals. The slit iris in the gecko is highly active. A plot of the measurements in Table I, given in Fig. 1, shows that below flash intensities ca. 0.0002 millilambert the critical intensity at given flash frequency is abnormally lowered. This implies that at these lower

<sup>13</sup> Cf. Hoagland, H., 1927-28, *J. Gen. Physiol.*, 11, 715.

<sup>14</sup> *Proc. Nat. Acad. Sc.*, 1936, 22, 17, 1937, 23, 23. *J. Gen. Physiol.*, 1936-37, 20, 211, etc.

<sup>15</sup> *J. Gen. Physiol.*, 1936-37, 20, 211, 363.

<sup>16</sup> Loeb, J., *Arch. ges. Physiol.*, 1891, 49, 175. Ehrenhardt, H., *Z. vergleich. Physiol.*, 1937, 24, 248. Öhm, J., *Arch. Ophth.*, Leipsic, 1931, 126, 547.

TABLE I

Mean critical flash frequencies ( $F$  per sec.) at fixed flash intensities (milli lamberts) for the gecko *Sphaerodactylus*. Flash cycle with light time ( $t_L$ ) equal to dark time ( $t_D$ ). Temperature =  $26.7^\circ \pm 0.06^\circ$ . At each point three observations were taken on each of 10 individuals, but not the same 10 individuals; the data are thus not truly homogeneous, in three instances ( $\log I = 2.5, 1.5, 1.5$ ) the differences between the means from duplicate tests exceed expectation. P.E.<sub>IF<sub>1</sub></sub> is the P.E. of the dispersion from which  $F_m$  is computed (see text).

$\log I$	$F_m$	P.E. <sub>IF<sub>1</sub></sub>
6.50	1.74	0.0988
	1.79	0.0454
5.00	2.62	0.0249
5.50	3.45	0.137
	3.46	0.0231
4.00	4.30	0.0695
4.50	5.33	0.153
3.00	6.94	0.102
3.50	8.69	0.124
2.00	11.22	0.0570
2.50	13.29	0.142
	13.93	0.118
1.00	15.60	0.453
1.50	17.47	0.530
	17.10	0.440
0.00	19.90	0.719
0.50	22.28	0.209
	21.70	0.168
1.00	23.16	0.447
1.50	24.20	0.642
	24.04	0.395
2.25	25.09	0.177

Mean critical flash intensities at fixed flash frequencies conditions otherwise the same as in the converse tests

$F$	$\log I_m$	$\log P.E._{IF_1}$
2.0	6.7475	7.1430
4.0	5.7551	6.0398
6.0	4.7137	5.0708
8.0	3.3043	3.6486
10.0	3.7523	4.0233
12.5	2.2353	4.6856
15.0	2.7898	4.7343
18.0	1.5032	3.6329
21.0	0.3648	2.6701
24.0	1.4839	0.1038

flash intensities the iris opens. This interpretation agrees with direct inspection, and can be checked by the closing of the iris with pilocarpine (Table II), this raises the critical intensity, in this region of the distortion of the graph, to a level which is theoretically predictable (see below). On the other hand, attempting to increase the disten-

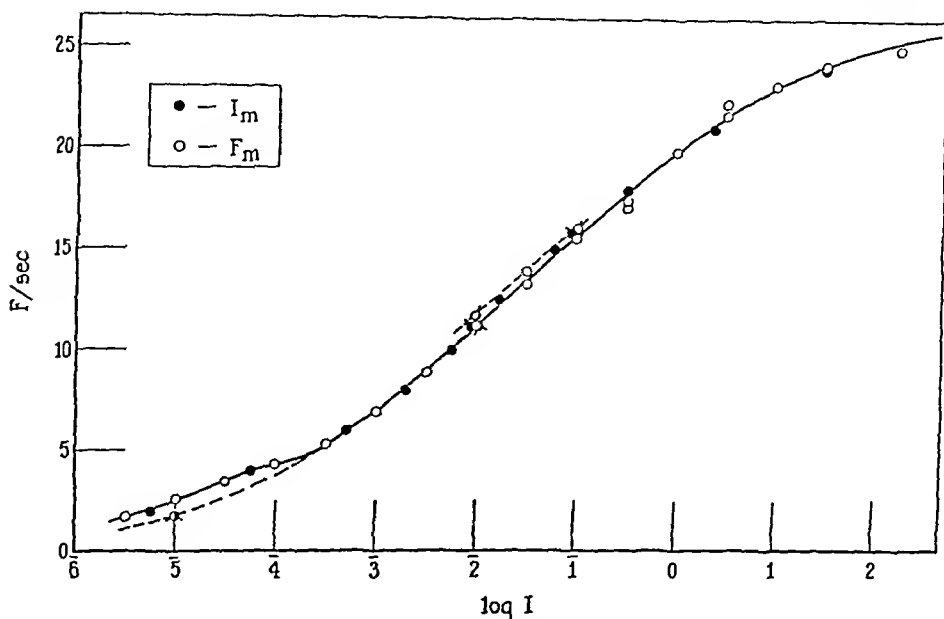


FIG. 1. Data in Tables I and II. The flicker response contour for the gecko *Sphaerodactylus*, temperature 26.7°, light time fraction in the flash cycle = 50 per cent. Below flash intensity = antilog  $\bar{4}.3$ , the expansion of the iris opening increases the effective intensity (see text), points obtained with iris constricted by pilocarpine (Table II) are distinguished by lower tags, this effect is checked by the action of atropine, ineffective in modifying the curve below  $\log I = \bar{4}.3$ , but increasing the critical flash frequency at higher intensities where pilocarpine is without effect, points obtained after instillation of atropine bear upper tags. Open circlets,  $F_m$ , solid circlets,  $I_m$ . The curve is obtained (cf Fig. 4) as described in the text, it is a probability integral.

tion of the iris opening by instilling atropine solution (Table II) in this part of the graph produces no real effect on the critical intensity. In the upper portion of the graph (cf Table II and Fig. 1), conversely, pilocarpine is without effect, but the opening of the iris under atropine lowers the critical intensity and raises the critical flash frequency.

The variation of  $I_1$  follows the rule uniformly found<sup>17</sup> in other cases.  $PE_{I_1}$  is directly proportional to  $I_m$  (Fig 2), and the proportionality constant is of the same order of magnitude as in other animals. There is no break in the plot of  $\log PE_{I_1}$  vs  $\log I_m$ , as in various other instances in which we deal with a single rather than a duplex population of elements of excitation.<sup>18</sup>

The variation of  $F_1$  (Fig 3), in correlation<sup>18</sup> with the low slope of the  $F - \log I$  curve (Fig 1), is low. It clearly tends to pass through a maximum, just beyond the inflection of  $F$  vs  $\log I$ ,<sup>18</sup> as in other cases. The relative scatter of  $PE_{F_1}$  is probably constant, as larger numbers of observations would make clearer. The

TABLE II

The effect of dilatation of the pupil of the gecko by *atropine* is to lower the critical intensity  $I_m$  for response to flicker at a fixed flash frequency  $F$ , or to raise the critical flash frequency  $F_m$  at a fixed flash intensity  $I$ . The normal values of  $F_m$  are taken from Table I, of  $I_m$  from the curve in Fig 1.  $I_m$  and  $F_m$  were obtained from sets of three observations on each of 3 individuals (see text), the  $PE$ s are for the dispersions. See Fig 1.

$\log I$	$F_m$	$F$	$\log I_m$	$\log PE_{I_1}$
2.00	11.74 $\pm$ 0.108 11.22 $\pm$ 0.0570	11.2	3.9437 2.00	4.8551
1.00	18.10 $\pm$ 0.211 15.60 $\pm$ 0.453	15.8	2.9428 1.03	3.5811

The result of constricting the pupil by *pilocarpine* is the reverse of the above and is more pronounced at the lower end of the  $F$  vs  $\log I$  curve.

3.00	1.781 $\pm$ 0.115 2.62 $\pm$ 0.0249	1.8	5.0082 5.500	7.4701
2.00	11.13 $\pm$ 0.147 11.22 $\pm$ 0.0570	11.2	2.0107 2.00	4.5430

extent of this scatter is influenced by the fact that the 10 individuals used were not the same for the different tests. This probably also accounts for the fact that the  $F_m$  points (Fig 1) do not clearly fall above<sup>15, 18</sup> the curve for  $I_m$ , as theoretically required and found for homogeneous data. In the analysis (Fig 4) of the  $F_m$  and  $I_m$  curves, however, it is clearly necessary to assign a slightly higher maximum  $F$  for the  $F_m$  data, as theory<sup>17</sup> requires.

<sup>17</sup> *J. Gen. Physiol.*, 1935-36, 19, 503; 1937-38, 21, 313, etc.

<sup>18</sup> *J. Gen. Physiol.*, 1938-39, 22, 311, 451; 1936-37, 20, 363; 1938-39, 22, in press.



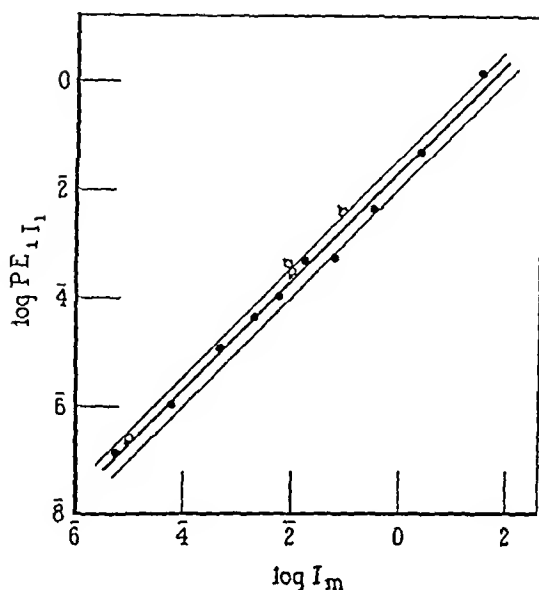


FIG 2  $\log P E_{I_{F1}}$  vs  $\log I_m$  The proportionality of the variation of critical intensity to mean critical intensity is simple and direct (the slope of the line—band—is unity) Data in Tables I and II, symbols as in Fig 1

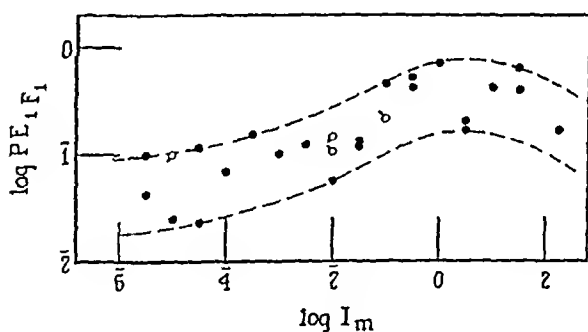


FIG 3  $P E_{I_{F1}}$  as a function of  $\log I$   $P E_{I_{F1}}$  is plotted on a logarithmic scale, since  $\sigma_{PE}$  is proportional to  $P E$  if the material is sufficiently homogeneous, and the vertical spread of the band in this plot should therefore be constant

#### IV

Taking into account the correction for the activity of the iris, considering that below  $\log I = 4.3$  the opening of the iris mechanically distorts the plot (Fig 1) by altering the intensity scale, we may test

the application<sup>19</sup> of the probability integral by means of the graph in Fig 4 For the  $F_m$  data (Table I),  $F_{max}$  is taken as 26.79, for the  $I_m$  measurements,  $F_{max} = 26.51$  The position of the points with iris constricted by pilocarpine is also indicated in Fig 4 The description of the data is clearly adequate The curve drawn in Figs 1 and 5

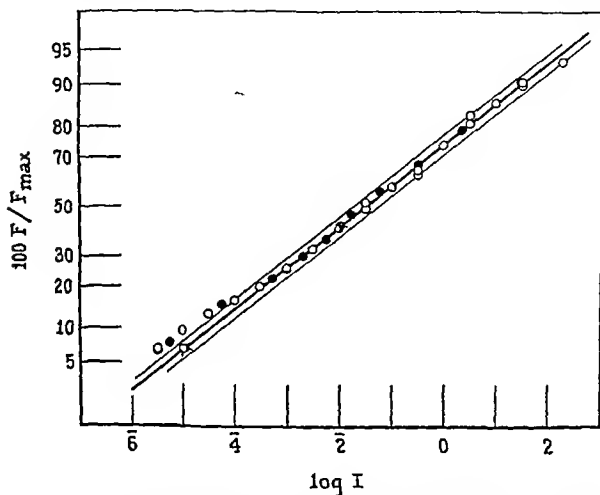


FIG 4 The data of Table I on a probability grid (see also Fig 1) Discussed in the text. For  $I_m$ , solid dots  $F_{max} = 26.51$ , for  $F_m$ , open circles,  $F_{max} = 26.79$  see text The deviation at the lower end, and the significance of the circles with tags, are discussed in the text and in the legend of Fig 1

is the average of the two very slightly different ones shown superimposed in Fig 4 (i.e., with the slightly different values of  $F_{max}$  each made equal to 100 per cent)

The analytical form of the  $F - \log I$  contour for the gecko is there

<sup>19</sup> *Proc Nat Acad Sc* 1937 23, 71, 1938, 24, 125 *J Gen Physiol* 1937-38 21, 17 1938-39, 22, 311, 451

fore the same as for other animals<sup>19</sup> Particular effort was made to detect any downward swing at the upper end of the curve, but within the limit of intensity afforded by the apparatus it does not materialize

The value of  $F_{max}$  is lower than any previously found for other dark adapted vertebrates, or for invertebrates This could result from the small size of the population of sensory units The inflection point in the curve comes at a level of  $\tau'$  which corresponds to that for the cone curves of vertebrates in general Comparison with the flicker contour for the turtle, *Pseudemys*, having purely cone retina,<sup>3</sup> is most clearly made by bringing the respective curves to the same value of  $F_{max} = 100$  per cent This does not change  $\tau'$ , and allows the comparison of the spread constants ( $\sigma'_{\log I}$ ) since the form of the function is the same in the two cases The data were obtained by the same procedure and with the same apparatus Under given conditions (*i e*, of light time cycle-fraction, and constancy of retinal area)  $F_{max}$  is specific for the type of animal and is not directly correlated with  $\tau'$  or  $\sigma'_{\log I}$  The comparison of the two curves on this basis is given in Fig 5

The gecko's flicker discrimination covers the same range of intensities as does that of the turtle The temperature differs—for the turtle measurements, 21.5°, for the gecko, 26.7° Using the temperature characteristic found<sup>3</sup> for the turtle, its curve is moved to the left to correspond to temperature 26.7° The respective abscissae of inflection ( $\tau'$ ) with this shift then differ by ca 0.08 unit The values of  $\sigma'_{\log I}$  differ by less than do those for the cone segments of the curves for different kinds of fishes or other vertebrates<sup>10 11</sup>

The largest rod curve we have thus far encountered is in the teleost *Fundulus*<sup>20</sup> In this case the descriptive parameters are  $F_{max} = 9.05$ ,  $\tau' = 5.06$ ,  $\sigma'_{\log I} = 0.66$  This is the closest approach we have to the curve for the gecko, and the differences (the particularly significant differences involve  $\tau'$  and  $\sigma'_{\log I}$ ) are in fact huge, the values for *Sphaerodactylus* are  $\tau' = 2.49$ ,  $F_{max} = 26.5$ ,  $\sigma'_{\log I} = 1.03$

The closeness of agreement of the parameters  $\tau'$  and  $\sigma'_{\log I}$  for gecko and turtle is, of course, accidental Data on two kinds of turtles would probably differ more But it is evident that these observations do not support the idea that a rod retina necessarily functions best

<sup>20</sup> *J Exp Zool*, 1939, in press

at low illuminations, even in a nocturnal animal. Nor is it indicated that a rod retina performs less ably than a cone retina at high illuminations. The danger of associating histological appearance and functional capacity in matters of visual performance is sharply emphasized.

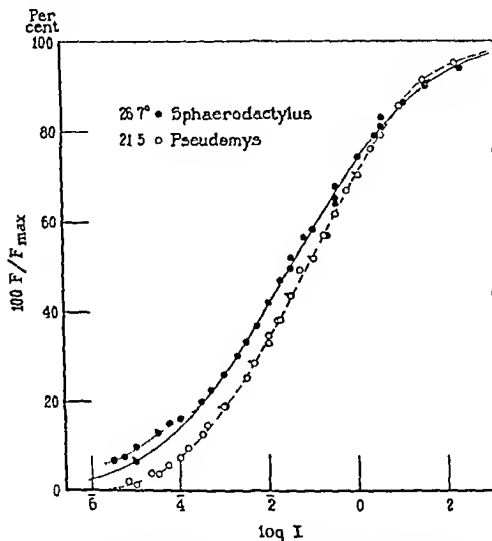


FIG. 5. The flicker response contour for the gecko (rod retina, at 26.7°) and for the turtle (cone retina), with  $F_{max} = 100$  per cent in each case.

The analysis of various visual functions among vertebrates demonstrates the presence of two constituent populations of sensory effects in many forms. These must be separately dealt with on the basis of their respective quantitative properties. The assumption that these two populations are directly due to the histological and chemical differentiation of the receptive units into rods and cones implies that

the quantitative properties of the data of visual performance in the various types of intensity discrimination are taken to be determined by quantitative properties of the rods and cones respectively. This assumption is arbitrary, unnecessary, and probably wrong.<sup>21</sup> The histological contribution to the assumption from the side of comparative properties of rods and cones in various animals is manifestly of small significance when the quantitative properties of visual performance in animals with purely rod and purely cone retinas are found to be essentially identical.<sup>22</sup>

#### SUMMARY

The flicker response contour for the gecko *Sphaerodactylus* (retina with only rods) agrees in all essential respects (intensity range, shape) with that for the turtle *Pseudemys* (cone retina), as determined under equivalent conditions with the same apparatus. With experimentally determined correction for the expansion of the iris at the very lowest intensities, the  $F - \log I$  contour for the gecko is a simple probability integral. Its maximum  $F$  is lower than that for other animals, this means simply a smaller number of available sensory elements. The quantitative parallelism in the magnitudes of the intensities at the inflection of  $F - \log I$  and the shape constants for rod and cone animals show that assumptions from comparative histological evidence concerning the properties of rods and cones in relation to visual performance may be quite misleading.

<sup>21</sup> *J. Gen. Physiol.*, 1938-39, 22, 341

<sup>22</sup> The difficulties of the situation cannot be escaped, logically, by suggestion that the gecko possesses a peculiar kind of retinal rod, this merely destroys the complex accepted conception of rod with which we started.

# THE ELECTRICAL IMPEDANCE OF MUSCLE DURING THE ACTION OF NARCOTICS AND OTHER AGENTS

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## INTRODUCTION

Since Bernstein first put forward the membrane hypothesis in 1900, the peculiar importance of the membrane in nerve and muscle function has been recognized. Because of their extreme thinness nerve and muscle fiber membranes have proved difficult to measure and to study directly.

With the electrical impedance method it is possible to analyze the electrical properties of these delicate structures under various conditions without injury to them. Höber (1912) in studying the conductivity of a suspension of erythrocytes, was the first to use high frequency impedance measurements on biological material. Fricke developed the theory of electrical impedance measurements and was the first to suggest an equivalent circuit for the living cell. More recently Cole has extended the measurements to marine eggs, muscle, nerve, and various other tissues. The success of the method as used by Cole and Curtis in determining changes in the electrical properties of *Nitella* and the squid giant axon during activity (Cole and Curtis, 1938, 1939) suggested that electrical impedance measurements of muscle might throw light upon the membrane changes which accompany the action of certain agents, especially narcotics.

Lillie (1922) reasoned that on the theory that normal excitability and conductivity of the irritable living system is determined by the properties of the surface film, one should expect that in narcosis the membrane would be rendered less alterable than before, i. e., stabilized. Hober (1907) held a similar view, and both agree that diminution of

permeability accompanies narcosis. However, more recently, an initial increase in permeability has been found to accompany narcosis in certain organisms under certain conditions (*e.g.* Seifriz, 1923, Hofler and Weber, 1926)

Inasmuch as electrical impedance measurements can be used as an indication of permeability of membranes to ions, it was decided to reinvestigate the question with this method

### *Material and Methods*

The sartorius muscle of *Rana pipiens* was used throughout

*Muscle Chamber*—The construction of the muscle chamber can be noted in Fig 1. Actually, three variations of this chamber were used in the course of the

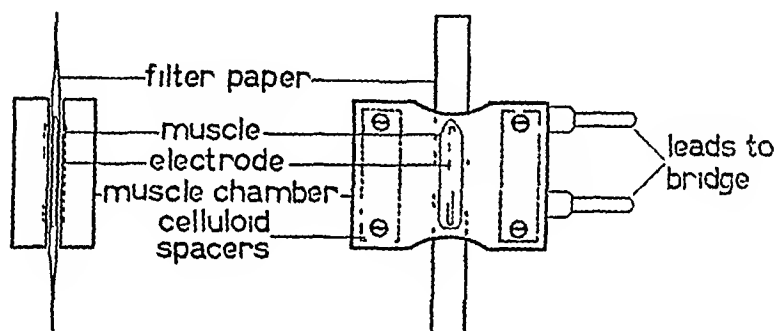


FIG 1 Cell for measuring transverse impedance of muscle, side and front view

experiments but they did not differ materially except for the size and shape of electrodes used

The chamber consists of two plates of bakelite separated by celluloid spacers of various thicknesses. The four brass screws which unite the two plates are insulated by bakelite cups in one model. In another model (in which there are round electrodes 1 mm in diameter) they are smeared with vaseline and situated quite a distance away from the electrodes.

The electrodes, which are of platinum black upon platinum, are flush with the surface of the bakelite plates. Copper wires are soldered to them and pass out of the cell through holes drilled in the bakelite. Where they emerge the copper wires are coated with picem cement to preclude the possibility of moisture seeping in to the copper-platinum junction.

The electrodes are replatinized for each experiment and are always kept moist. The excellent reversibility of the reactions studied shows that no significant amount of the agents used upon the muscle is adsorbed by the platinum black in such a way that it cannot easily be washed away.

As has been mentioned three types of electrodes were employed (a) small round electrodes 1 mm in diameter, (b) larger discs 15 mm in diameter, and (c) long strip electrodes 20 mm in length and 1 mm wide. The first type was found to be unsatisfactory inasmuch as its small size does not permit parallel lines of current flow. The long electrodes (c) were most satisfactory from the point of view of parallel current flow. As the length of the electrodes is increased the central component of the current flow, which is perpendicular to the fiber axes of the muscle becomes increasingly important and the end components, where the current flows out into the tissue beyond the ends of the electrodes and the lines of current flow are not parallel become less important. It is much easier to analyze transverse impedance measurements where the lines of current flow are mostly parallel and perpendicular to the muscle and for this reason the muscle chamber containing the strip electrodes 20 mm long and 1 mm wide was the one finally selected for these studies although the others were used in preliminary work.

To give an example of the type of difficulty which was encountered when using the small electrodes (1 mm in diameter) it was found impossible to determine with these electrodes the resistance of the moist strips of filter paper which were used for circulation of solutions past the muscle. To be specific, with the small electrodes the resistance of a muscle covered with one strip of filter paper seemed to be greater than the resistance of a muscle covered with two strips of filter paper (one on each surface). This was obviously a problem to be investigated. The difficulty disappears when larger electrodes are used. In other words, when longer electrodes are used, the total resistance of muscle plus filter papers increases as filter papers are added, as is to be expected.

The reason for the anomalous results with small electrodes is that with small electrodes most of the lines of current flow are not parallel. The electrical set up is a complicated one which may be expressed by saying that it results in a situation in which the resistances of muscle and filter papers are not in series with one another.

*Solutions Used*—The composition of the Ringer's solution used in these experiments was as follows: 7 gm NaCl, 0.3 gm KCl, 0.25 gm  $\text{CaCl}_2$  in a liter of solution. The solution was buffered to pH 7.4 with sodium bicarbonate.

All salt solutions and sugar solutions used were isosmotic with frog blood as calculated from depression of freezing point data.

Ringer's solution to which a small quantity of narcotic had been added was used in some experiments. These solutions are not strictly isosmotic with frog blood but the amount of narcotic added was in every case quite small and the departure from the isosmotic condition was not considerable.

In practically all cases the pH of the solutions was adjusted to 7.4 (the pink of phenol red) with sodium bicarbonate. It was not found necessary to control the temperature.

*Method of Circulation*—Solutions are circulated past the muscle by means of two moist filter paper strips completely covering the muscle and placed one on



each side between the muscle and each electrode (Fig 1) The dimensions of the filter paper strips are approximately 5 mm by 10 cm, their width being approximately the same as the width of the muscle

Solutions are conducted through them to the muscle very rapidly If a second solution of a different conductivity from the one which has been circulating is introduced, an impedance change is noted almost immediately in the head phones Also, solutions seem to be washed out from filter paper strips as quickly as they enter

The rate of drip of the solutions was kept fairly constant from experiment to experiment, 1 cc of solution being delivered in about 35 seconds

Whether the filter papers run along the muscle (parallel to the long axis of the muscle) or across the muscle (at right angles to the long axis of the muscle) seems to have no measurable effect upon either (1) the final equilibrium resistance value when a new solution is circulated or (2) the time constant (the term "time constant" will be used throughout to mean the time interval elapsing between the beginning of the reaction and the time when half the effect has taken place)

When it is realized that the muscle volume is only about 0.084 cc and the "sugar space" (which, as will be proven later, is intercellular space) is only about 0.021 cc and that the volume of solution delivered in a few seconds is much greater than the sugar space, it is understandable that it should make practically no difference whether the filter papers are placed along or across a muscle Also, diffusion into the muscle from the point of contact with the solution is probably very rapid

This filter paper method of changing solutions is much to be preferred to one method used by Osterhout (1922) in measuring the conductivity of *Laminaria* When changing solutions Osterhout took his measuring cell apart, removed the tissue, washed the tissue in the new solution, and replaced it in the measuring chamber In the experiments here reported, the handling of the tissue and variations in pressure attendant upon the reassembling of the cell were eliminated Also, continuous uninterrupted reading during diffusion of the new solution into the tissue was possible

The electrical properties of the filter papers are not altered by the solutions which are circulated It was found that the ratio of the resistance per filter paper to the specific resistance of the solution circulated is practically the same if Ringer's solution or a solution of half isosmotic sugar and half Ringer's solution (50 per cent sugar) is used

	Ringer's solution	50 per cent sugar
<u>Resistance per filter paper</u>		
<u>Specific resistance of solution</u>	0.092	0.088

In many cases the muscle was tested at the end of the experiment and was found to be excitable

### Apparatus

The alternating current Wheatstone bridge used was devised by Cole and Curtis (1937) and has been described by them. The current sent through the measuring cell is so small that the impedance was independent of it. Transverse impedance was measured at all times. It was measured as parallel resistance,  $R_p$ , and capacity  $C_p$ .

With the substitution method used in these experiments, an accuracy of 0.5 per cent was obtained.

The equivalent series resistance  $R$  and the series reactance,  $X$  were calculated from the formulae

$$R = \frac{R_p}{1 + (R_p C_p \omega)^2} \quad X = \frac{R_p^2 C_p \omega}{1 + (R_p C_p \omega)^2}$$

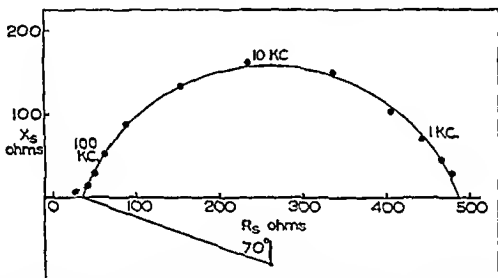


FIG 2 Frequency impedance locus,  $X$  = series reactance,  $R$  = series resistance,  $X$ , for transverse measurements of frog sartorius muscle in Ringer's solution. Frequencies indicated are in kilocycles per second.

In the earlier experiments complete frequency runs were made from 200 cycles to 1000 kc (0.2 kc, 0.5 kc, 1 kc, 2 kc, 5 kc, 10 kc, etc.). When it was noted, however, that the experimental points on the resulting impedance locus gave so good a fit (Fig. 2) it was decided for the sake of speed and simplicity to determine but three frequency points experimentally,  $X$  = 1 kc, 10 kc, and 100 kc, since three points determine a circular arc. Inasmuch as speed was of considerable importance in some of the experiments where impedance measurements were made during the action of a chemical agent, this procedure was found to be a great convenience.

In some experiments dealing with the effect of chemical agents upon electrical impedance over long times, impedance measurements were made at 1000 cycles only. It might at first seem preferable to make d.c. measurements in order to

follow changes of membrane resistance. However, D C measurements cannot give any indication of capacity variations which may be taking place

### *Theory and Calculations*

The theoretical muscle may be considered to be a uniform random distribution of parallel, circular, cylindrical fibers in a medium (Bozler and Cole, 1935, Cole and Curtis, 1936). Then the specific resistance of the muscle perpendicular to the fiber,  $r$ , the resistance of the medium,  $r_1$ , the resistance of the fibers,  $r_2$ , and the volume concentration of the fibers in the medium,  $\rho$ , bear the following relationship to one another

$$\frac{r/r_1 - 1}{r/r_1 + 1} = \rho \frac{r_2/r_1 - 1}{r_2/r_1 + 1}$$

The above equation is analogous to the Maxwell equation for the conductivity of a suspension of spheres

It has been shown that the muscle is the electrical equivalent of a circuit containing at least two resistances and one variable impedance element of constant phase angle (Bozler and Cole, 1935). If the series resistance of such a circuit,  $R_s$ , is plotted on the abscissa and the series reactance,  $X_s$ , is plotted on the ordinate, for different frequencies, the circle diagram or impedance locus, which has long been used in communication engineering and which was introduced by Gildemeister and by Cole to express biological data, is obtained. Theoretically, it would be expected that the center of the circular arc representing the impedance locus of such a circuit would lie on the resistance axis, i.e., that the phase angle would be  $90^\circ$ . It is found experimentally for muscle that the center of the circle is below the resistance axis and that the phase angle is less than  $90^\circ$  (Fig. 2). Cole interprets this to mean that the single variable impedance element in the circuit has dielectric loss.

On the assumption that the membrane is non-conducting (as will later be shown to be the case) it is possible to solve for  $\rho$  in the following manner

$$\frac{1 - \frac{r_1}{r_0}}{1 + \frac{r_1}{r_0}} = \rho$$

where  $r_0$  is the extrapolated zero frequency resistance Then

$$r_2 = \frac{r_0 r_\infty - r_1^2}{r_0 - r_\infty}$$

where  $r_\infty$  is the extrapolated infinite frequency resistance

### *Discussion of Results*

It was decided to investigate first the effect of simple inorganic cations upon the electrical impedance of muscle When the 1000 cycle resistance of a muscle treated with certain cations, *i.e.* Ba and Ca, was plotted against time it was found that the curve describing the resistance changes consisted of two parts (Fig 3) It was thought that perhaps the first part of the curve represented merely penetration of the cation into the intercellular spaces of the muscle and the second, interaction of the cation with the muscle tissue In order to investigate this possibility, the effect upon electrical impedance of the diffusion into the intercellular spaces of the muscle of a substance which was known not to react with the muscle substance and to be, physiologically speaking, rather inert was studied Mixtures of Ringer's solution and isosmotic sugar solutions were used for this purpose When the effect of diffusion into the intercellular spaces alone upon the electrical impedance of muscle had been quantitatively determined it was then possible to continue the studies and to investigate the effect of complex organic narcotics

#### *Effect of Inorganic Cations upon Electrical Resistance of Muscle*

The effect of inorganic cations upon the 1000 cycle resistance of frog sartorius was studied in the following manner All solutions used were isosmotic with frog Ringer's solution and were circulated past the muscle by means of strips of filter paper as described above Chlorides of sodium and potassium and of the alkali earths barium, calcium, and magnesium were used It is important that all solutions used be strictly isosmotic with frog blood inasmuch as varying osmotic pressure seems to have a profound influence upon the electrical resistance of muscle as can be seen from an experiment where 50 per cent, 120 per cent, and 200 per cent Ringer's solution was employed By a 50 per cent Ringer's solution is meant a solution containing one part of Ringer's solution to one part of distilled water, while by a

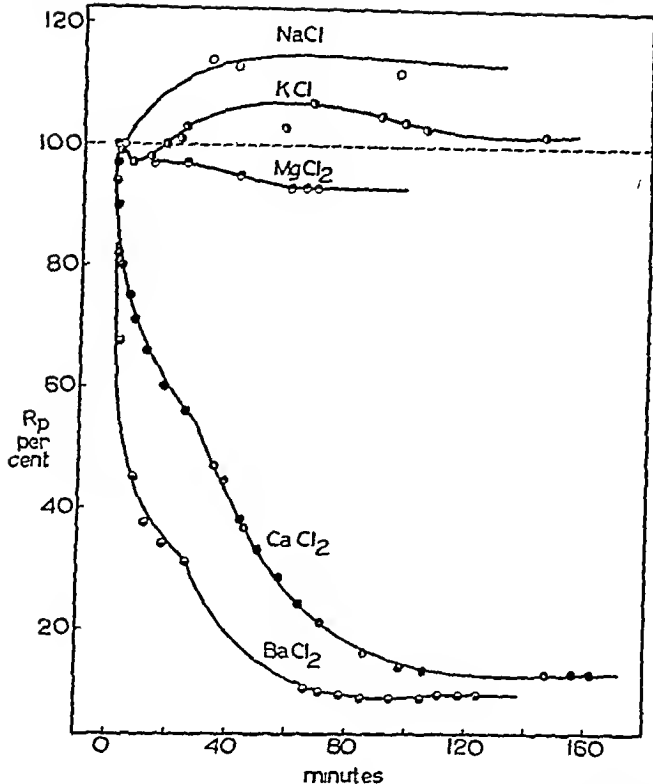


FIG 3 Thousand cycle resistance of muscle, expressed as percentage of equilibrium value in Ringer's solution, *vs* time in minutes after substitution of test solutions for Ringer's solution. Typical experiments. All solutions isosmotic with Ringer's solution. Resistance of isosmotic chloride solutions compared with resistance of Ringer's solution. NaCl, 101 per cent, KCl, 96 per cent, BaCl<sub>2</sub>, 81 per cent, CaCl<sub>2</sub>, 87 per cent, MgCl<sub>2</sub>, 91 per cent.

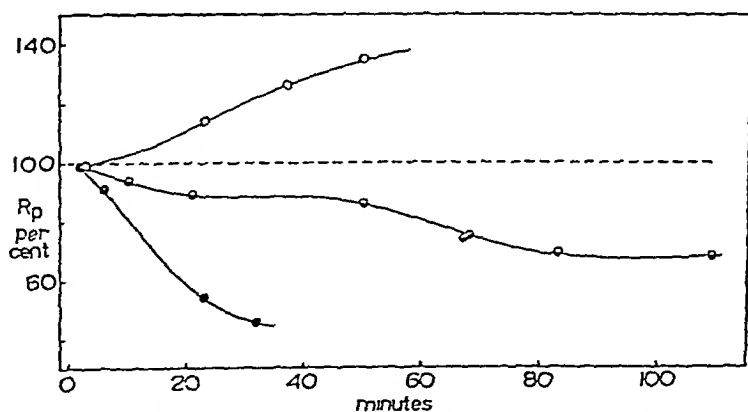


FIG 4 Influence of Ringer's solutions of various osmotic pressures upon resistance of muscle. Thousand cycle resistance of muscle, expressed as percentage of equilibrium value in pure Ringer's solution, *vs* time in minutes after substitution of test solution for Ringer's solution. Test solutions are 50 per cent Ringer (O), 120 per cent Ringer (◐), and 200 per cent Ringer (●).

200 per cent Ringer's solution is meant a solution in which the salts are dissolved in one half of the usual amount of distilled water. In Fig 4, percentage resistance change is plotted against time. It will be noted that a hypotonic solution causes a marked rise in resistance while hypertonic ones cause a drop. This is, of course, to be expected since the conductivities of these solutions differ from that of Ringer's solution. It should also be noted, however, that the muscle has been found to follow Boyle's law and act as a simple osmometer when treated with Ringer's solutions of different osmotic pressures (Chao, Chiao, and Chi, 1938).

The effect of inorganic ions upon 1000 cycle resistance of muscle can be seen in Fig 3, where the percentage change in resistance (the resistance in Ringer's solution being taken as 100 per cent) on addition of the solutions is plotted against time. Addition of the isosmotic chloride solution was not begun until the muscle had reached a steady resistance value in Ringer's solution. If not treated, this equilibrium value may be maintained for many hours. Each curve represents a typical experiment. In all, fourteen experiments of this type were done.

In every case the difference in conductivity between Ringer's solution and the isosmotic chloride solution should be taken into consideration in estimating the percentage change. The resistances of the solutions are listed under Fig 3. (The differences in conductivity could not be corrected for in the graph since the rate of penetration of the solutions was not known.)

The resistance of a solution of  $MgCl$  isosmotic with frog Ringer's solution was found to be 91 per cent of that of pure Ringer's solution. Thus the 10 per cent drop in resistance of the muscle after treatment with isosmotic  $MgCl_2$  is not significant, and we may say that isosmotic  $MgCl_2$  has no effect upon the 1000 cycle resistance of sartorius muscle. Also, addition of  $NaCl$  and  $KCl$ , respectively, seems to have little effect upon the resistance of muscle. Isosmotic  $KCl$  raises the resistance 1 or 2 per cent during the course of about 2 hours and  $NaCl$  raises it a little more (about 10 per cent). The small effect of  $NaCl$  and  $KCl$  is surprising inasmuch as these substances cause marked contracture in muscle (Gasser, 1930). Isosmotic solutions of  $BaCl_2$  and  $CaCl$ , on the other hand, markedly affect muscle resistance, causing it to drop.

A slight degree of recovery (as measured by increase in resistance in the direction of the normal value) was obtained in these experiments after the muscle had been allowed to remain in the isosmotic  $\text{BaCl}_2$  and  $\text{CaCl}_2$  solutions for as long as 2 or 3 hours. If solutions containing one part of isosmotic alkali or alkali earth chloride solution and four parts of Ringer's solution are used, the normal resistance is maintained for hours.

The relative lack of effect of pure isosmotic solutions of  $\text{NaCl}$ ,  $\text{KCl}$ , and  $\text{MgCl}_2$  upon the muscle, insofar, at least, as these effects are mirrored in impedance changes is of interest. Since the work of Sidney Ringer, the importance of balanced salt solutions and the toxicity of solutions of single pure isosmotic salts have been stressed again and again. Since impedance measurements are usually (*cf.* experiments with narcotics reported in this paper, for instance) a delicate means of determining physiological membrane changes, and toxicity has been associated with breakdown of membrane resistance, it might be supposed that pure solutions of  $\text{Na}$ ,  $\text{K}$ , and  $\text{Mg}$  would affect muscle resistance markedly, yet this is not the case.

In this connection it might be interesting to refer to observations which will be published shortly on injury potentials of the non-myelinated nerve of the spider crab, where it was found that pure isosmotic solutions of the chlorides of the earth alkalis  $\text{Ba}$ ,  $\text{Ca}$ ,  $\text{Sr}$ , and  $\text{Mg}$  have no effect upon the magnitude of the injury potential when applied to the uninjured portion of the nerve. Inasmuch as substances which are ordinarily considered toxic cause the injury potential to fall or, if strong enough, completely disappear, when applied to the uninjured part of a nerve, one would expect pure isosmotic solutions of the earth alkalis to have the same effect if they were truly toxic.

In general then, it may be said that while changes in osmotic pressure of solutions affect muscle impedance greatly, the use of certain salt solutions which are not physiologically balanced does not seem to do so. Some cause other than the fact that they are "pure solutions" and "unbalanced" must be found to account for the strong action of isosmotic  $\text{CaCl}_2$  and isosmotic  $\text{BaCl}_2$ .

It is dangerous to generalize concerning the action of ions upon electrical resistance of tissues and membrane permeability. Osterhout

found in most cases, from observations on *Laminaria* and other similar forms, that monovalent ions cause electrical resistance to fall and that bivalent ions cause it to rise and then fall (Osterhout, 1922) However, the work of others on different types of tissue (e g Hogben and Gordon, 1930) as well as the results of these experiments make a generalization inadvisable

It will be noted that there is a distinct break in the curves representing the influence of isosmotic  $\text{BaCl}_2$  and  $\text{CaCl}_2$  upon the resistance of muscle (Fig 3) The break was encountered in every experiment where these solutions were used It was thought that possibly the first part of the curve represented penetration of the substance into the muscle and the second interaction between the substance and the muscle As has been stated above, it was in order to investigate this question, as well as for other reasons that it was decided to study the question of penetration in connection with some substance which was believed to be relatively inert as far as interaction with living tissues is concerned

### *Experiments with Sugar*

*Why Sugar Solutions Were Used*—It has been established (Overton, 1904) that although isosmotic sugar solutions cause loss of irritability, the sugar itself is not toxic, but the effect is produced as a result of the absence of intercellular electrolytes If one part of Ringer's solution or isosmotic  $\text{NaCl}$  is added to as much as three or four parts of isosmotic sugar solution, no loss of irritability results Such a solution may be said to be physiologically inert, and yet it has a conductivity very different from that of pure Ringer's solution It seemed to be an ideal type of solution to use for penetration studies, inasmuch as if the resistance of the muscle in Ringer's solution is first determined and the specific resistance of the sugar solution is known, the electrical resistance of a muscle treated with the sugar solution will throw some light on the penetration of sugar or exit of electrolytes from the intercellular spaces By observing changes in electrical resistance we should be able to study the time course of diffusion, reversibility of diffusion, and similar problems Penetration of the substance into the interior of the muscle fibers, if any, the effect of the substance upon the integrity of the fiber membranes, and like problems may



be investigated if resistance and capacity to an alternating current are measured over a wide frequency range

*Sugar-Ringer Mixtures Do Not Alter the Tissue*—In order to be certain that mixtures of Ringer's solution and isosmotic sugar solutions are truly inert and do not alter the tissue in any way, *i.e.* that they merely penetrate the intercellular spaces and do nothing else, the following experiment was performed

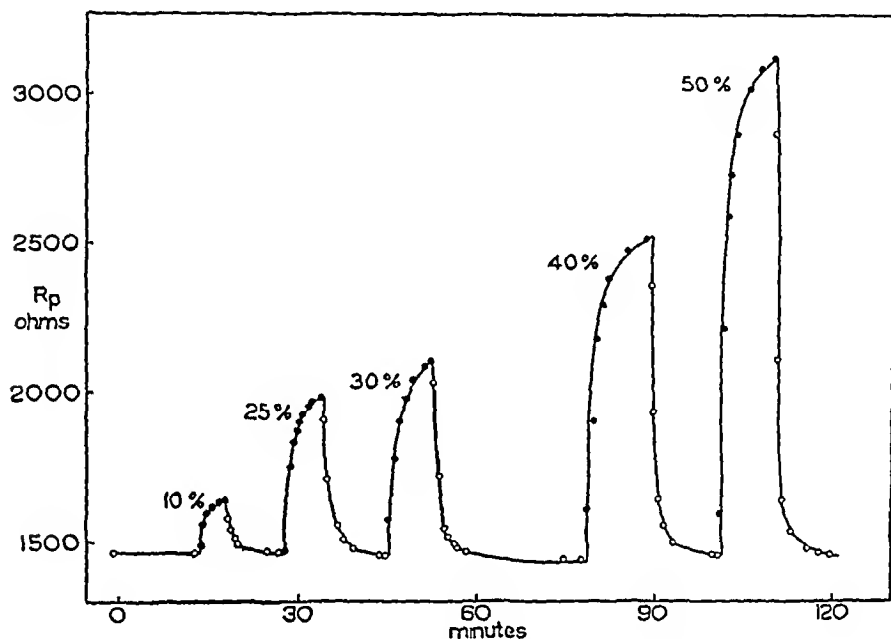


FIG 5 Thousand cycle resistance of muscle in various concentrations of isosmotic dextrose-Ringer mixtures *vs* time in minutes. Percentages indicate concentration of dextrose in dextrose-Ringer mixtures. O, muscle in Ringer's solution, ●, muscle in Ringer-dextrose mixture. Typical experiment. All data obtained on same muscle. Note almost perfect reversibility.

A muscle which had attained an equilibrium resistance value in Ringer's solution was treated successively with various concentrations of Ringer's solution-isosmotic sugar solution mixtures, and recovery in pure Ringer's solution was permitted after treatment with each concentration of sugar (Fig 5). Reversibility of resistance changes due to treatment with sugar was nearly perfect. This makes it difficult to believe that the sugar solutions cause serious leakage of salts from the muscle fiber interiors.

Besides, it was found that the ratio

$$\frac{\text{1000 cycle resistance of muscle}}{\text{Specific resistance of solution}}$$

for the muscle in Ringer's solution and in each concentration of sugar remains remarkably constant. In the experiment represented in Fig. 5, the mean is 19.8 with a variation of only  $\pm 0.33$  from the mean. This indicates that at 1000 cycles the fiber membranes are practically non-conducting, at least as far as can be detected by these measurements, and that the sugar space is the intercellular space. By entirely different experimental procedure, Urano and Fahr have shown that the electrolytes replaced by sugar are intercellular electrolytes (Urano, 1908, Fahr, 1909).

When the percentage of Ringer's solution in the Ringer sugar mixtures used is plotted against the reciprocal of the resistance of the muscle in that mixture, the data fit a straight line passing through the zero point fairly well (Fig. 6). This would seem to indicate that what we are measuring is the amount of Ringer's solution present in the intercellular spaces, and, again, that the fiber membranes are non-conducting at 1000 cycles.

*Sugar Ringer Mixtures Do Not Cause Swelling or Shrinking*—When the volume concentration,  $\rho$ , is calculated from the data furnished by such an experiment, it is found to remain constant when varying concentrations of sugar are used to bathe the muscle, indicating that sugar of these concentrations (10 to 50 per cent) causes no swelling nor shrinking of the fibers. If sugar solutions of these concentrations did cause swelling or shrinking, their effect would increase with increasing concentration, and  $\rho$  would not be similar for all of them.

*Rapidity of Diffusion*—Diffusion of sugar into muscle (or exit of electrolyte from the intercellular spaces) is quite rapid, half the effect being over, when two filter paper strips are used, in about three quarters of a minute. If sugar were able to penetrate the fiber membranes, the rapidity of diffusion would, of course, be greater. Inasmuch as narcotics, as will later be shown, do penetrate fiber membranes, the value obtained with sugar sets an upper limit to the time required for penetration of narcotics through muscle.

In Fig. 7, 1000 cycle resistance is plotted against time for two paired muscles treated with 40 per cent sugar (isosmotic sugar solution

Ringer's solution as 4 6), in one case the muscle being in contact with two filter papers (one on each side) and in the other, with only

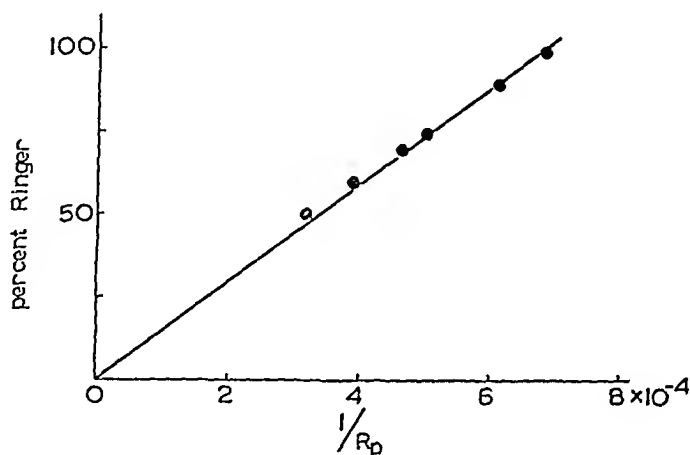


FIG 6 Reciprocal of equilibrium value of thousand cycle resistance of muscle in various concentrations of isosmotic dextrose-Ringer mixtures *vs* percentage of Ringer in dextrose-Ringer mixture Data from same experiment as is represented in Fig 5

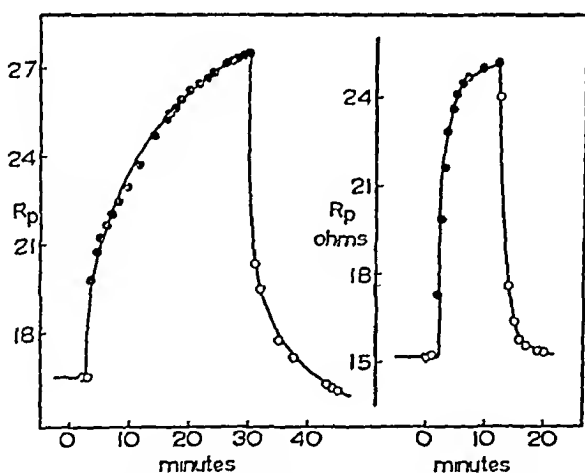


FIG 7 Thousand cycle resistance of muscle in Ringer's solution (O) and in 40 per cent dextrose, *i e* isosmotic dextrose solution Ringer's solution as 4 6, (●) *vs* time in minutes On left, solutions circulated past muscle by means of one filter paper, on right, solutions circulated past muscle by means of two filter papers, one on each side of muscle Same muscle in both experiments On left, equilibrium (1 per cent change in 3 minutes) attained in 23 5 minutes, on right, in 6 5 minutes

one filter paper. It will be noted that the total rise in resistance after treatment with sugar is about the same for both muscles, but that the time necessary for this resistance change to be brought about is four times as long when only one filter paper is in contact with the muscle as it is when two filter papers are in contact with the muscle, one on each side. Theoretically of course, this result is to be expected since when there are two filter papers, not only need a substance diffuse only half as far into the muscle, but it is diffusing in from both surfaces simultaneously.

The ratio of the times of rise in the two cases, 1:4, is to be expected if the process follows the well known diffusion equation which states that the amount of substance diffusing in is proportional to the square root of the time. This equation has been shown to hold for the diffusion of lactic acid and oxygen through muscle (Eggleton, Eggleton, and Hill, 1928) and for the diffusion of inorganic phosphate through muscle (Stella, 1928).

*Additional Data Obtained from Sugar Experiments*—Values for  $\rho$ , the volume concentration of fibers,  $r_2$ , the resistance of the interior of the fibers, and  $\theta$ , the phase angle of the impedance locus, were calculated for the muscle in Ringer's solution and in the sugar mixtures according to the equations quoted above (*cf.* Theory and calculations).

The volume concentrations in Ringer's solution vary from 75 per cent to 81 per cent with an average of 77.7 per cent. The  $r_2$  values in Ringer's solution vary from 176 to 310 with an average of 231 ohms specific resistance, which is about 3.2 times that of Ringer's solution. The phase angle,  $\theta$ , was found to average  $71.0^\circ$ .

These values of  $\rho$ ,  $r_2$ , and  $\theta$  compare favorably with those obtained for frog sartorius by Bozler and Cole (1935). They obtained volume concentrations of 77.3 per cent and 76.2 per cent and  $r_2$  values of 264 and 253 for two muscles, and found the phase angle to average  $71^\circ$ . Schulze (1927), using a somewhat different method, obtained a volume concentration of 86.1 per cent.

It will be noted that with low concentrations of sugar, *e.g.* 25 per cent, the volume concentration remains constant, as does the fiber interior resistance value. In other words, it seems that dilute sugar solutions do not cause swelling or shrinking or penetrate into the

fibers, so far as can be detected by our methods. When higher concentrations of sugar are used, however, we find a different situation. In 50 per cent sugar (one experiment) the volume concentration seems to remain practically the same as in Ringer's solution, but the resistance of the interior of the fibers increases about twofold. Two experiments were done with a 75 per cent sugar solution. It is difficult to tell whether this solution causes membrane permeability to change or shrinking of fibers to take place. At any rate the resistance of the fiber interiors increases about two and one-half times.

The findings just enumerated, however, although interesting, are by-products of the sugar experiments. The rapidity of the resistance changes on treatment of the muscle with sugar solutions indicates that penetration alone cannot account for the small break in the resistance curve which was always encountered when the muscle was treated with Ba or Ca, and suggests that intercellular penetration need not be taken into consideration when studying the effect of organic narcotics.

Once the troublesome question of the order of magnitude of impedance changes due to penetration between the fibers alone had been dealt with, it was possible to begin the study of the effect of the more complex substances.

### *Effect of Narcotics upon Muscle Impedance*

The following group of substances, each of which has some narcotic action, surface active behavior, or lipoid solubility was studied: saponin, chloroform, sodium taurocholate, butyl and amyl alcohol, iso-amyl carbamate, chloral hydrate, and sodium salicylate. All of these were found to depress 1000 cycle resistance of muscle when used in sufficient quantity.

Similar results were obtained for *Laminaria* and for frog skin with ether, alcohol, chloroform, and chloral hydrate by Osterhout (Osterhout, 1922). Gildemeister (1920) studied, by measuring the inductance necessary to neutralize it at 1000 cycles, the capacity of frog skin and muscle during the action of various agents.

An analysis of the effect upon 1000 cycle resistance of muscle of increasing doses of iso-amyl carbamate is presented in Fig. 8, where resistance is plotted against time. It will be noted that minute changes in concentration alter the character of the influence of the

drug upon resistance As the concentration of the drug is increased, the rise in resistance is followed by a gentle fall in resistance On increasing the concentration still further, the rise in resistance is momentary and the depression of resistance more marked It has been known for some time that dilute solutions of narcotics have an effect different from that of higher concentrations This is brought out quantitatively by the electrical resistance experiment just de

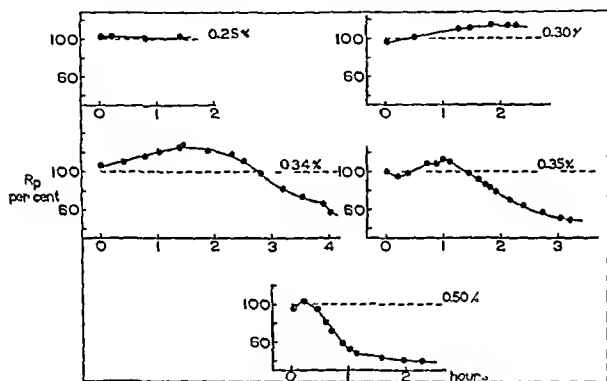


FIG 8 Influence of various concentrations of iso amyl carbamate upon resistance of muscle Thousand cycle resistance of muscle expressed as percentage of equilibrium value in pure Ringer's solution, vs time in hours after substitution of the drug in Ringer's solution for pure Ringer's solution Five different muscles Percentages indicate amount of the drug dissolved in Ringer's solution

scribed Besides it is possible in such an experiment to follow the effect of the narcotic with time and to establish that the action of a dilute narcotic solution over a long time resembles that of a higher concentration acting for a shorter time

Since dilute solutions of narcotics result in anesthesia, while higher concentrations are toxic, these results suggest a correlation of resistance increase with anesthesia, and resistance decrease with toxicity

On a somewhat different basis, Osterhout (1922) working with

alcohol and ether on *Laminaria agardhii*, was led to a similar conclusion. He found that the initial rise in resistance encountered when using these agents was reversible, while the subsequent drop in resistance below normal was not. This led him to believe that anesthesia, which is by nature inherently reversible, is associated with permeability decrease, while the subsequent irreversible toxicity on continued action of the narcotic is associated with permeability increase.

It was felt that perhaps information from impedance data over a wide frequency range and long times could throw light upon which aspects of the electrical characteristics of the cell, *i e*, resistance of the medium, of the fiber interior, resistance, and capacity of the membranes were affected by narcotics.

Cole and Curtis (1938) have just found for *Nitella* that on excitation there is a 200-fold decrease in membrane resistance and a 15 per cent decrease in membrane capacity, while in the giant fiber of the squid (Cole and Curtis, 1939) there is a 30-fold decrease in membrane resistance and a 2 per cent decrease in membrane capacity. It was hoped that an analysis similar to theirs might throw some light upon the mechanism of narcosis.

Cole and Curtis have shown that on theoretical grounds, if the membrane resistance alone were to change, the impedance locus at a single frequency would describe a circular arc which is tangent to  $r_{\infty}$  and has its center on a line perpendicular to the resistance axis and running through  $r_{\infty}$  if  $\theta$  is unchanged. If, on the other hand, the membrane capacity alone were to change, the impedance values at any one frequency would follow along the familiar frequency impedance locus.

It was found that during the action of narcotics the impedance follows almost perfectly the path predicted for a change in membrane resistance alone (Figs 9 and 10). With weaker drugs, *e g* 0.34 iso-amyl carbamate (Fig 10) the circular arc describing the impedance change with time does not actually pass through the  $r_{\infty}$  point, but if extrapolated, it would. With stronger solutions, *e g* a solution of Ringer's solution saturated with chloroform (Fig 9), the arc actually does pass through the  $r_{\infty}$  point.

In other words, narcotics break down the membrane resistance progressively and, if strong enough, and permitted to continue their

action long enough, completely Membrane capacity is only very slightly affected by narcotics

In every case when a drug was first applied there was a short initial increase in membrane resistance This must not be overlooked since it may be associated with the initial narcotizing action

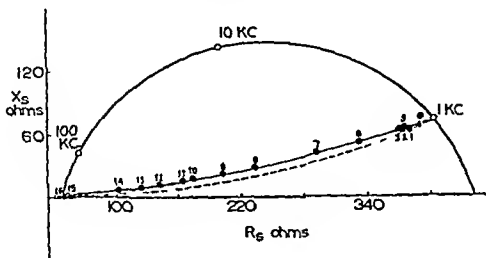


FIG 9 Frequency impedance locus,  $\circ$  series resistance,  $R$ , vs series reactance,  $X$ , for muscle in Ringer's solution ( $\circ$ ) Frequencies given are in kilocycles per second The time locus ( $\bullet$ ) shows how the impedance changes at 1 kc when the muscle is bathed with Ringer's solution saturated with chloroform Numbers indicate order in which data were taken Broken line is a circular arc tangent to the resistance axis at the  $r_0$  point

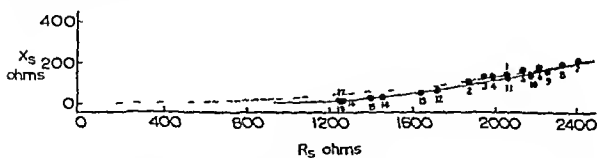


FIG 10 Time impedance locus,  $\circ$  series resistance,  $R$ , vs series reactance,  $X$ , for muscle in 0.34 per cent iso-amyl carbamate in Ringer's solution at 1 kc. Broken line is a circular arc tangent to the resistance axis at the  $r_0$  point.

of the anesthetic, while the decrease in membrane resistance on longer action may be linked to toxicity This is all the more probable since the initial increase in membrane resistance is more pronounced when a lower concentration of narcotic is used than when a strong dose is employed (Fig 10)



## DISCUSSION

In connection with the results here obtained with narcotics, it would be interesting to ascertain the effect of possible swelling or shrinking of the muscle fibers upon the impedance of muscle. Secondly, proof that the changes which have been ascribed to the membrane are, indeed, due to changes in the fiber membranes (plasma membranes) and not to changes in the fibrillae, the mitochondria, or other cell inclusions, is also necessary.

*Swelling and Shrinking* —As to the first point, if swelling or shrinking takes place on application of a drug, and swelling or shrinking affects impedance, the circle diagram should be shifted to the right or to the left, and the extrapolated infinite frequency resistance should be different when the muscle is in Ringer's solution or in the drug. This, however, is not the case (Fig 11), the extrapolated infinite frequency resistance during drug action coincides with the extrapolated infinite frequency resistance during treatment with Ringer's solution.

It was thought that this question might be further studied by determining in the following manner whether the sugar space,  $z_c$  the intercellular space, is altered during the action of a drug.

A muscle which had first attained equilibrium in Ringer's solution was treated first (*a*) with a dilute solution of a drug dissolved in Ringer's solution, secondly, after equilibrium had again been attained (*b*) with a solution of one part of a concentration twice as great of the drug dissolved in Ringer's solution plus one part of isosmotic sugar solution and then thirdly (*c*) with the dilute solution of drug dissolved in Ringer's solution again. Since it had already been established that 50 per cent sugar does not cause appreciable swelling nor shrinking of the fibers, it was felt that the sugar space might remain unaltered if the drug itself causes no swelling or shrinking and a dilute concentration of drug is used.

However, this method was not very satisfactory inasmuch as the action of the drug often continued when solution (*b*) was used and may have prevented the resistance from reaching the value it would ordinarily have reached with 50 per cent isosmotic sugar alone.

In some cases the ratio

$$\frac{R_0}{R_1} z_c = \frac{\text{Resistance of muscle in medium}}{\text{Resistance of medium}}$$

was practically constant for solutions (a) and (b) but this constancy seems to be the result of a delicate balance of just the correct con-

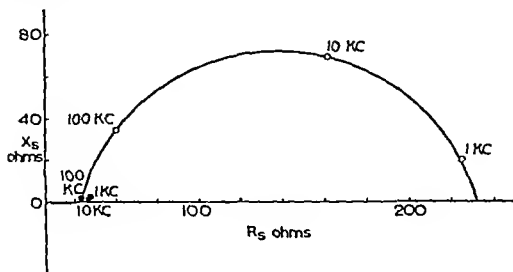


FIG 11 Frequency impedance loci,  $\circ$  series resistance,  $R_s$ , vs series reactance,  $X_s$ , for muscle in Ringer's solution ( $\circ$ ) and in Ringer's solution saturated with chloroform ( $\bullet$ ). Frequencies are given in kilocycles per second

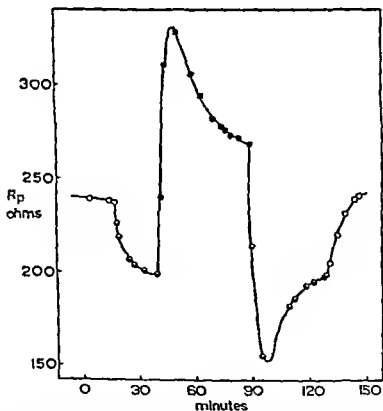


FIG 12 Thousand cycle resistance of muscle in Ringer's solution ( $\circ$ ), in 1-400 Na salicylate dissolved in Ringer's solution ( $\bullet$ ), and in one part 1-200 Na salicylate dissolved in Ringer's solution plus one part isosmotic sucrose solution ( $\circ$ ) vs time in minutes

centrations In the cases where the ratio  $R_0 - R_1$  did not remain constant for solution (a) and solution (b), it was always noted that after maximum resistance was attained in solution (b) the curve representing the resistance sloped downward (Fig 12) This would suggest that the action of the drug continues in solution (b) inasmuch as no decline was ever encountered when sugar and Ringer's solution alone were used

It is possible that some swelling or shrinking takes place when the various narcotics mentioned above are used However, it is highly improbable that swelling or shrinking can account for the entire effect of narcotics upon muscle impedance

*Is the Impedance of the Fiber Membranes Being Measured?*—It has been assumed all along that the complex impedance locus is the locus of the impedance of the plasma membrane and that the changes in the overall muscle impedance are due to changes in membrane impedance It might be well to examine now the arguments which support this assumption

Hober's experiments on the conductivity of erythrocytes (Hober, 1912), which have already been mentioned, suggest strongly that it is the cell membrane which offers impedance to alternating current He found that the conductivity of erythrocytes to alternating currents of nine million cycles per second is about the same as that of a solution of disintegrated cells at any frequency Living cells have a lower conductivity than dead ones That this is due to the presence of cell surfaces rather than to the cell as a whole is shown by the above type of experiment

The simplicity of the complex impedance locus of muscle (Fig 2) favors the view that there is only one type of structure which is offering resistance to the measuring current That this single structure is the plasma membrane is probable since Cole has found that a circle diagram describes the complex impedance locus for a great variety of cells and tissues

An argument which favors the view that it is the cell membrane rather than the membranes of the fibrillae which has been measured in these experiments on muscle, is the following If it is the fiber membranes which are the structures offering impedance to the electric current, the calculated fiber membrane capacity is of the order of  $10 \mu\text{f}/\text{cm}^2$  (Cole and Curtis, 1936), if it is the membranes of the

fibrillae, their membrane capacity would have to be about  $20 \mu\text{f}/\text{cm}^2$ . Inasmuch as practically every type of living material measured has been found to have a membrane capacity in the neighborhood of  $1 \mu\text{f}/\text{cm}^2$ , it is highly probable that it is the fiber membrane which is being measured here.

### CONCLUSIONS

Cole and Curtis (1938) consider that the membrane capacity represents the ion impermeable aspect of the membrane and the membrane resistance, the ion permeable aspect. In the case of narcotics, we have an example of the independence of these two postulated aspects of the membrane. Here one, the ion permeable, alone is affected. This same aspect of the membrane is affected in excitation in *Nitella* and the squid giant nerve fiber (Cole and Curtis, 1938, 1939). In fertilization of marine eggs, on the other hand, the non-conducting, ion impermeable part of the membrane appears to be changed (Cole and Spencer, 1938). It would seem reasonable to suppose that narcosis and injury, on the one hand, would have a greater resemblance to excitation, on the other, than either would have to the mechanism behind fertilization. This supposition seems to be supported by impedance data.

That, in general, membrane resistance is a less stable aspect of the membrane than is membrane capacity, also seems to be brought out by these experiments on the effect of narcotics upon muscle impedance.

I am greatly indebted to Prof. K. S. Cole for much indispensable advice and technical guidance during the course of this work, to Dr. H. J. Curtis for his generous assistance at all times, and to the Biological Laboratory at Cold Spring Harbor for the facilities so kindly put at my disposal during the preliminary experiments.

### SUMMARY

1 The effect of certain inorganic cations upon the electrical impedance of the sartorius muscle of the frog was investigated. While Na, K, and Mg have little effect upon the resistance of muscle, Ba and Ca cause it to fall. The use of physiologically "unbalanced" salt solution does not in itself seem to affect muscle impedance.

2 The time course of the effect upon muscle impedance of the

penetration of substances into the intercellular spaces was studied by treating the muscle with sugar solutions. Half of the effect is over in three-quarters of a minute when the sugar solution is permitted to circulate past both sides of the muscle. This sets an upper limit for the time necessary for inorganic cations and organic narcotics to reach the cell surfaces. The action of inorganic cations and organic narcotics upon muscle is slow compared to the time necessary for them to reach the scene of action.

The penetration of the sugar solutions into the intercellular spaces of muscle was found to follow the well known diffusion law, the amount diffusing in being proportional to the square root of the time.

Average values of 77.7 per cent for  $\rho$ , the volume concentration of fibers, 231 ohms specific resistance for  $r_2$ , the resistance of the interior of the fibers, and  $71.0^\circ$  for  $\theta$ , the phase angle of the impedance locus, were obtained for the muscle in Ringer's solution. How these values change when the muscle is placed in various concentrations of sugar was also studied.

3 The action of a number of organic narcotics upon muscle was studied. All decrease 1000 cycle resistance if the concentration is sufficiently high. A detailed analysis of the action of the narcotic, iso-amyl carbamate, was made, and it was noted that low concentrations increase resistance while higher concentrations decrease it.

By investigating the effect of narcotics upon muscle impedance over a wide frequency range, it was found that during narcosis the resistance of the fiber membranes first increases and then decreases, and, if the drug is present in sufficiently great concentration, membrane resistance may completely disappear. Membrane capacity is only very slightly affected.

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# THE VISIBILITY OF SINGLE LINES AT VARIOUS ILLUMINATIONS AND THE RETINAL BASIS OF VISUAL RESOLUTION

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## I

### *Nature of Problem*

The present measurements were made to explore the problem of why visual acuity varies with illumination. This question is not limited to the human eye, nor to the vertebrate eye (*cf.* Chrenhardt, 1937) but is a general one since even insects (Hecht and Wolf, 1929, Hecht and Wald, 1934) and crabs (Clark, 1935) show a similar dependence of visual acuity on illumination. The matter has been extensively studied in man (Uhthoff, 1886, Koenig, 1897, Hecht, 1928) merely because the measurements are conveniently made and the results are of common interest. The data for all organisms show that the capacity for resolving visual detail increases in a definite way with the intensity of illumination.

A quantitative explanation of this phenomenon was suggested several years ago (Hecht, 1928, Hecht and Wald, 1934), and is based on the supposition that the receptor elements in the eye vary in sensibility over a wide range of light intensities, so that their thresholds are distributed in the usual manner of populations. This statistical distribution need not be a fixed property of the individual elements, but may be variable in time (Hecht and Wolf, 1929) depending perhaps on the recovery of the individual elements following stimulation as suggested by Berger and Buchthal (1938 *b*). In terms of this distribution the number of elements functional in a given retinal area increases with the illumination, and since the resolving power of a receiving surface varies with the number of active elements per unit



area, it follows that the resolving power or visual acuity of the eye should increase as the retinal illumination increases

This explanation requires no knowledge of the distribution of light in the image formed on the retina, and hence does not concern itself with the actual nature of visual resolution. However, the appearance of the image on the retina in terms of diffraction optics is certainly an important factor in visual resolution (Hartridge, 1922, Shlaer, 1937). We therefore thought that a detailed study of it may point the way toward a formulation of the relation between visual acuity and illumination in terms which are more concrete than the statistical one already proposed.

The ordinary test objects used in visual acuity measurements are unit configurations like letters, hooks, or broken circles. These are complicated patterns and a calculation of the exact light distribution in their retinal images is almost impossible. Therefore, it is difficult to determine precisely what these test objects measure (*cf.* Berger, 1936, Berger and Buchthal, 1938 *b*). A simpler test object is an evenly spaced grating. However, because this is a repeating pattern, limits to its resolution may be set by factors other than intensity distribution (Abbe, 1873, Shlaer, 1937). The simplest unit test object is a single opaque line against a uniformly illuminated background, and the light distribution produced by it on the retina may be determined in complete detail in terms of diffraction optics (Rayleigh, 1903, Hartridge, 1922). We therefore measured the relation between brightness and visual resolution using such a test object, in order to describe as nearly as possible what happens at the retina.

## II

### *Method and Apparatus*

The apparatus is shown diagrammatically in side view in Fig. 1. A large sheet of flashed opal glass *G*, selected for uniformity, is illuminated by a short focus lantern-slide projector *LCP*, to furnish a surface of uniform brightness. The opal plate is outlined as a disc 2 feet in diameter by an opaque shield *S*, and serves as the background for the test object. The test objects are wires *W* of different thickness, the finer wires are of drawn tungsten, while the coarser are brass rods. Each wire is separately mounted across the diameter of a brass ring *R*, which is slightly larger than the illuminated background. Three hooks *H* in the

opaque shield hold the brass ring in place in front of the illuminated background, the brass ring may be rotated in its own plane, so that the wire occupies any selected direction

The brightness and color of the illuminated background is varied by a series of neutral and color filters  $F$  placed immediately in front of the projection lens  $P$ . The maximum brightness is just above 30 millilamberts. With the proper Wratten filters the brightness may be decreased in steps of almost any size down to any value desired. A heat absorbing glass  $A$  is placed between the condensers in order to protect the gelatin filters from the heat of the projection system, this renders the light slightly greenish.

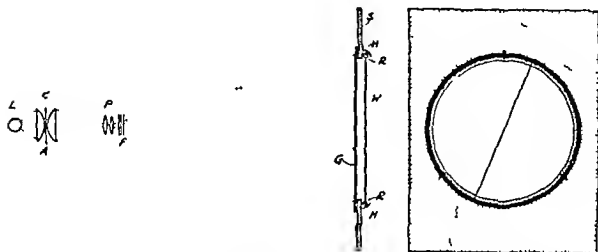


FIG 1 Diagram of the optical arrangements for visual resolution of a single line against a uniformly illuminated background.  $LCP$  is a lantern slide projector with its lamp  $L$ , condensers  $C$ , projection lens  $P$  and heat absorbing glass  $A$ . Neutral filters  $F$  control the illumination on the flashed opal glass  $G$ . The opaque shield  $S$  outlines the field in front of which is the wire  $W$  set in the ring  $R$  and held by the hooks  $H$ . the sketch at the right shows how these are arranged from the observer's point of view.

The lamp and projector are in an anteroom with the projection lens  $P$  just protruding through an opening into a small dark room. The opal glass and opaque shield are in the doorway between this small dark room and another larger dark room in which the observer is. The part of the apparatus facing the observer is shown at the right in Fig 1. When the projection lamp is on the observer does not see the brass ring; he sees only the illuminated circular surface and the wire in front of it. The observer sits in a chair on rollers which moves over the floor near a scale so arranged that the position of his eyes at any moment may be read from a pointer attached to the chair and in contact with the scale on the floor.

Before making measurements the observer stays in the dark for about 20 minutes. He is then presented with the lowest illumination to be investigated,

in front of which the wire has been placed in one of three specific positions by the recorder. The observer adapts to this illumination for a few minutes, after which he moves towards the wire until he can describe its direction with certainty. All observations are binocular, with the natural pupil. Three settings are usually made with each intensity, and the observer always adapts for a few minutes to each intensity before beginning a determination. The procedure is continued until the whole intensity range is studied, and usually takes about an hour and a half.

TABLE I

*Relation between Background Light Intensity and the Visual Angle Subtended by an Opaque Line When It Just Becomes Visible*

Light intensity in millilamberts	Visual angle in minutes		
	Series 1	Series 2	Series 3
0 00000447	16 14		
0 00000603		11 09	10 52
0 0000214	4 94		
0 0000263		4 09	3 85
0 0000676	2 40	2 10	2 10
0 000295	1 50	1 32	1 21
0 000692	1 167	0 703	
0 00151			0 721
0 00302	0 454	0 340	
0 00457	0 368	0 275	0 328
0 0200	0 139	0 104	0 118
0 0603	0 0867	0 0569	0 0684
0 263	0 0365	0 0295	0 0321
0 661	0 0191	0 0158	0 0168
2 95	0 0154	0 0109	0 0118
6 92	0 0130	0 00912	0 00875
30 2	0 0112	0 00802	0 00785

The influences of the distance from the test object (Freeman, 1932) and of the size of surround (Lythgoe, 1932, Hecht and Smith, 1936) are eliminated by keeping the final resolution distance of the observer between 2 and 3 meters from the test object. This is done by using wires between 12  $\mu$  and 8 mm in thickness.

### III

#### *Measurements*

We made three series of measurements, all with S H as observer and E U M as recorder. Series I was exploratory, and involved

three separate runs, we used such wires as were easily available and determined the general relation between angular size and brightness. After this, some of the wires were changed and the light source was put on alternating current and its voltage kept constant. We then made series II which also consisted of three independent runs. Finally, several weeks later, after further changes in the wires, and

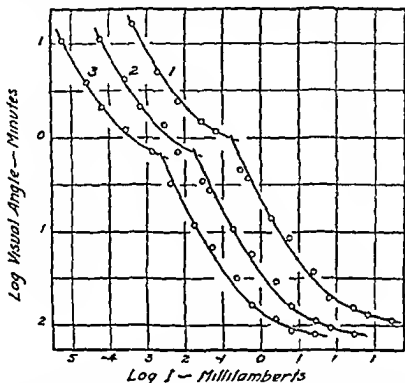


FIG 2 The relation between the brightness of the background and the visual angle subtended by the thickness of the wire when it just becomes resolved against the illuminated background. Series 1, 2, and 3 are shown separately, the points for series 3 are in their correct positions on the coordinates while series 2 and 1 are moved to the right 1 and 2 log units respectively. Note the separate relations shown by the low intensity measurements and the high intensity ones, corresponding to rod and cone functions respectively. The six curves drawn through the data represent the theoretical equation  $\alpha = b[1 + (1/KI)^{1/2}]$  in which  $\alpha$  is the visual angle,  $I$  the light intensity, and  $b$  and  $K$  are two constants.

a minor change in the filter arrangements we made the third series of measurements consisting as before of three separate runs. The averaged measurements for the three series are given in Table I. It is apparent from Table I that both the light intensity and the just resolvable visual angle corresponding to it vary over a large range, for convenience of examination, therefore, the data are plotted in Fig. 2 on a double logarithmic grid.

## IV

*Rod and Cone Vision*

Perhaps the most obvious property of the measurements as shown in Fig 2 is their natural separation into two sections, one at the lower brightnesses, and the other at the higher brightnesses. By now this is a familiar phenomenon, and a similar division has been found in almost all visual functions when they have been measured over a wide intensity range (Hecht, 1937*a*). It is practically certain that the low intensity section represents the behavior of the rod system in the retina, while the section at high intensities represents the behavior of the cone system, in accordance with the duplicity theory first suggested by Schultze (1866) and later developed by von Kries (1929) and Parnaud (1881).

In this connection we made some preliminary measurements using violet light instead of white. In terms of the relative spectral sensitivities of the rods and the cones, it is to be expected that with violet light the separation of the two segments of the data will be greater than with white light. This is true for dark adaptation, flicker, intensity discrimination, visual acuity, and instantaneous threshold (*cf* the review by Hecht, 1937*a*). Our measurements, though too few to be recorded here, show the same phenomenon. The low intensity section for violet is about 1 log unit to the left as compared to that for white light, while the high intensity sections are, of course, in the same position for both. We may therefore safely conclude that the two sections of the data in Fig 2 correspond to the histological and functional duality of the vertebrate eye as expressed by the duplicity theory.

It is to be noted in Fig 2 that the fifth point from the right is above the curve in each of the three series. This is true for each run made, and is a real phenomenon. We measured this point with a variety of combinations of wires, distances, and filters, but it always came out slightly to one side of the line, as if at this intensity there is some change in retinal function. However, the point is not correlated with any subjective phenomenon, and its meaning is obscure.

## V

*Extent of Resolving Power*

One of the striking things which the measurements show is the great range of resolution of which the eye is capable. At the lowest light intensities the eye can just see a line whose thickness subtends a visual angle of about 10 minutes, while at the highest intensity the just resolvable line subtends only 0.008 minute which is very nearly 0.5 second of arc. This is a range of about 1 to 1200, and is 10 or 20 times the range ordinarily found with test objects like a broken circle, a hook, or even a grating. With such objects the resolution at the lowest light intensities is usually between 20 and 30 minutes of arc, while at the highest intensities it is between 0.5 and 1 minute, thus covering a maximum range of only 1 to 60.

This difference in range shown by the two kinds of test object occurs mainly in the upper light intensities. At low brightnesses, the two yield values of the same order of magnitude, at high intensities the resolution with single lines attains a value about 60 times as great as with a hook or with a grating.

It is worth noting that these differences in the resolving power of the retina between a single line and a grating are not confined to the human eye. Recently Ehrenhardt (1937) has measured with a lizard the relation between the intensity of illumination and the visual acuity. With a grating made of equally wide spaces and bars the resolution at the lowest intensity corresponds to a visual angle of very nearly 1 degree, while at the highest intensities it corresponds to an angle of 11.5 minutes, this is a range of about 1.5. However, with a grating made of spaces 30 times wider than the bars, the resolution at the lowest light intensities is about 30 minutes, while at the highest intensities it corresponds to about 1.3 minutes, making a range of 1.25. Note here too that at low light intensities the just resolvable visual angle is much the same for both types of test object, and that the difference in range is the result of the better resolving power for widely spaced lines at the high light intensities.

A similar situation holds for the bee's eye. With a grating of equal bars and spaces, the highest resolution corresponds to an angle

just equal to the smallest ommatidial separation (Hecht and Wolf, 1929) On the other hand, a single bar can be resolved even when it subtends an angle only a quarter as wide as an ommatidium (Buddenbrock, 1935)

## VI

*Maximum Resolution and Intensity Discrimination*

The values obtained at the highest intensities deserve closer examination At a maximum brightness of 30 millilamberts we could resolve with certainty a wire which subtends only 0.5 second of arc However, Fig. 2 shows clearly that a slightly smaller angle would probably be resolved at still higher brightnesses As it is, 0.5 second is a much smaller angle than the minimum of 3 to 4 seconds found by Hartridge (1922) for single lines A number of individuals in the laboratory confirmed this small angle, most people having no difficulty in achieving it (*cf.* also Shlaer, 1937) We attribute this to the evenness of the illuminated background, the just resolvable angle increases rapidly when the background is irregularly illuminated For this reason we gave up very early the efforts to make the measurements with the open sky as background

The geometric image on the retina of the wire corresponding to the highest resolution is barely  $0.04 \mu$  wide Since the central cones of the fovea are between 2.0 and 2.6  $\mu$  in diameter (Rochon-Duvigneaud, 1907, Østerberg, 1935), the geometrical image is only about 1/60 of the width of a single cone

However, as Rayleigh (1903) and Hartridge (1922) have pointed out, such computations of retinal distances are meaningless, since the image on the retina is too small to be described in the simple terms of geometrical optics Because of diffraction at the pupil, the image of a wire is not a sharp black shadow covering only a small fraction of a cone, but a fine fuzz of a shadow extending over several cones The distribution of light under such conditions has been carefully worked out by Rayleigh (1903), and though tedious to compute, is straightforward and simple

Measurements of the pupil size, made during the course of our work showed that the pupil never was smaller than 3 mm in diameter even at the highest illuminations We have therefore used this

value in the computation of light distribution on the retina in terms of diffraction. It is significant in this connection that visual acuity, though it varies sharply with pupil diameter below 3 mm, is practically constant for pupils larger than 3 mm (Lister, 1913, Cobb, 1915, Berger and Buchthal, 1938a). The constancy of visual acuity for pupils larger than 3 mm is probably due to the neutralization of two tendencies of the light distribution in the retinal image as the result of diffraction the image becomes better as the pupil increases, while as the result of chromatic aberration it becomes worse as the pupil increases (Harttridge, 1918, 1922).

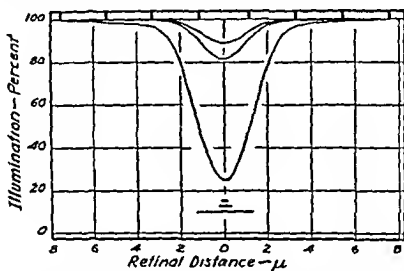


FIG 3 Light intensity distribution in the diffracted retinal images produced by three wires whose geometrical images are shown as three lines in the lower part of the figure. The pupil is taken as 3 mm. The scale on the top of the figure represents the retinal mosaic, consisting of cones whose diameters are  $2.3 \mu$ , this being the average size of the group of cones in the very center of the fovea.

We have computed the light distribution in the retinal image of a series of wires, using Rayleigh's equations and a pupil of 3 mm. As an example, Fig 3 shows the results for three lines whose geometrical images are  $0.3$ ,  $0.5$ , and  $2.6 \mu$  wide, corresponding to visual angles of  $4.1$ ,  $6.8$ , and  $35.5$  seconds respectively. The distribution corresponding to the smallest resolvable angle ( $0.5$  second) is so flat and near the upper edge of the ordinates that it can hardly be shown in a drawing of this scale. However, it has the same form as the others, and its vertical dimensions are a constant fraction of those of the larger distributions. Indeed, this is true of nearly all the sizes, except only the largest wires. The curves in Fig 3 give the light intensity



at any point in the retinal image, the region below any curve means light intensity while the region above any curve means shadow or absence of light. The scale at the top is marked off in units of  $2.3 \mu$  representing the average cone separation in the fovea. By determining the area of light under the curve for each cone one can compute the relative amount of light falling on each cone.

Hartridge (1922) first made a computation of this sort for a wire subtending 8 seconds of arc. He used a pupil of 2 mm and a cone width of  $3.2 \mu$ , and found a difference of 10 per cent between the light on the central cone and the ones on either side. He chose an angle of 8 seconds for computation because of Rayleigh's theoretical expectation that this is the minimum resolvable. However, he actually found a value of about between 3 and 4 seconds for the resolution of a black line against an illuminated white surface, which would make the difference in light on two adjacent rows of cones more nearly 5 per cent. Even so, this is a fairly coarse intensity difference, and it is hard to see how it can be the limiting factor in visual acuity. One cannot explain this by recalling that intensity discrimination becomes poorer with small areas (Lasareff, 1911, Heinz and Lippay, 1928, Steinhardt, 1936), because small areas are not involved in such measurements. To be resolved at these fine angles, a line must be long, a great many times longer than it is wide, in other words, the number of retinal elements involved is large, and corresponds to areas such as yield the best values for intensity discrimination.

The present situation is indeed different from that envisaged by Hartridge. The best modern histological measurements (Rochon-Duvigneaud, 1907, Østerberg, 1935) show the central cones of the human retina to be between 2.0 and  $2.6 \mu$ . Moreover, our best resolution with a 3 mm pupil corresponds to 0.5 second. When the computation for intensity distribution in such a retinal image is carried through for these data, we find a much more critical condition. Assuming the general illumination on the retina to be 100 per cent, then it comes out that a central row of cones  $2.3 \mu$  wide is illuminated by 98.83 per cent of the prevailing intensity, while the row to either side has a light intensity of 99.78 per cent. The difference in light intensity between the two rows is 0.95 per cent, and is a value near those usually found in measurements of intensity discrimination at

high light intensities. It corresponds to a value of  $\Delta I/I = 1/105$ , and is exceeded only by Aubert's (1865) best value of  $1/146$  and Helmholtz's (1866) value of  $1/167$  at the highest illuminations. For more moderate illuminations like our maximum the values generally range around  $1/100$ .

This computation tells us that a fine line is recognized at such small angles because even its fuzzy and extended shadow reduces the light on one long row of cones to a value which is just perceptibly less than the light on the row of cones on either side of it. The line appears sharp because it produces a perceptible shadow on one row of cones only. Note that the row of cones to either side of this critical row has its illumination reduced by only 0.22 per cent compared to the prevailing illumination next to it, and that this small difference in intensity cannot be perceived, it would mean a  $\Delta I/I$  of about  $1/400$  and cannot be achieved by the eye. Thus when a line becomes recognizable, the light distribution which it produces on the retina is such that only one row of cones is just perceptibly shaded by it.

From Hartridge's original computation it was not clear why a line is perceived as a sharp line instead of as a band of shadow gradually fading at the edges, and it has been necessary to assume some central nervous mechanism for converting such a gradual distribution into a sharp line. However, in view of our present computation the line appears sharp simply because its diffracted image on the retina affects only one row of cones differently from the rest. Thus Hartridge's idea that the maximum resolving power of the eye is determined by its capacity for intensity discrimination is even better than originally anticipated.

We have supposed that in the retina the comparison is made between the row of perceptibly shaded cones and the row immediately next to it. This makes an intensity difference of just 0.95 per cent between the two rows. However, in view of the nature of intensity difference perception (Hecht, 1935), it is even more likely that what is recognized is the intensity difference produced on the central row of cones from the time it is affected by the general retinal illumination to the time when it is affected by the shaded center of the image, and these successive intensities are achieved by the obvious eye movements made during the effort to resolve the line in the field. Considered

this way, the intensity difference is about 1.17 per cent, which is also near the minimum perceptible intensity difference, but which leaves room for perception of the still smaller differences which must correspond to the better resolution shown by Fig. 2 as possible at higher illuminations than those we used

## VII

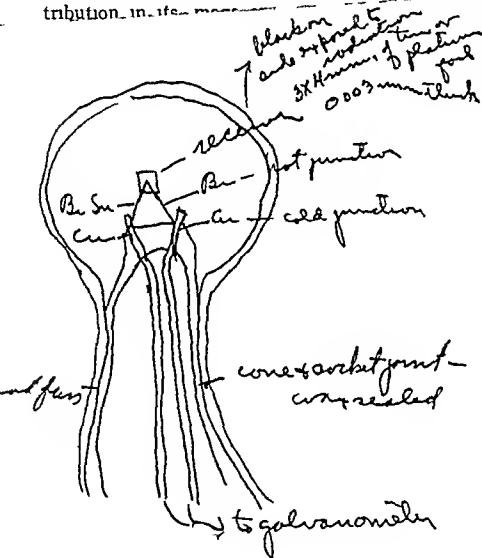
### *Visual Resolution at Different Illuminations*

If the highest resolving power of the eye is determined by its maximum capacity for intensity discrimination in the diffracted retinal image, is it not possible that the resolving power of the eye at any illumination is also determined by the intensity discrimination of the retina at that illumination? One may put this more directly. The essential point of the explanation of maximal visual resolution is that because of the transformation of the optical image by diffraction, the limiting factor in detail recognition is the capacity of the eye for intensity discrimination. It is well known that the capacity for intensity discrimination varies with the light intensity, therefore the visual resolution of detail must also vary with the light intensity.

The distribution of light in the diffracted image of a single line depends on the pupil size and on the width of the line, but not on the intensity of the light. Therefore with a fixed pupil, the distribution depends on the width of the line, which at once sets the depth of shadow in the diffracted image, and fixes the light intensity on the central row of cones as compared either with that prevailing generally on the retina or with that on the row of cones to either side of the central row. The fractional difference in light intensity produced on the retina is thus a fixed property of a given size of line, and has a specific value  $\Delta I/I$ , where  $I$  is the background intensity and  $\Delta I$  is the difference between it and the intensity on the shaded central row of cones. Since the just perceptible fractional intensity difference  $\Delta I/I$  has a different value for each light intensity (Aubert, 1865, Koenig and Brodhun, 1889, Hecht, 1935), the line will be resolved only at intensities which are equal to or greater than the value of  $I$  for which the particular fraction  $\Delta I/I$  is the just perceptible one. This is for a fixed size of line and a variable intensity.

A similar situation exists for a fixed light intensity  $I$  and a test object of variable size. The fixed value of  $I$  has, corresponding to it, a minimum fractional intensity difference which the retina can just recognize. If now the opaque line produces an intensity distribution in its image

in a fractional intensity difference  $\Delta I/I$  then the intensity difference will be resolved, if  $\Delta I/I$  is greater than the critical  $\Delta I/I$  then the intensity difference will be perceptible



is necessary to be extended by the intensity difference it may be done by producing a geometrical image. The data is in the intensity difference will vary only with the geometry of the object. It is possible to distinguish the shape of objects  $0.5 \mu$  and cones receive the central shadow. Thus by changing the shadow falling on the eye. But the resolution corresponds to the general resolution of cones

(11)

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... is proportional to it. In other words, the fractional intensity difference  $\Delta I/I$  produced on the retina by these fine wires is, as a first approximation, directly proportional to the

visual angle they subtend. Therefore, if the limiting factor in visual resolution is intensity discrimination, then the relation between the just resolvable visual angle and the light intensity should be the same as the relation between the just discriminable fraction  $\Delta I/I$  and the light intensity.

The essential condition for this derivation is that while the diffracted image of the wire extends over 5 or more cones, its geometrical image covers only a fraction of a cone. This condition holds over nearly the whole of the range of visual angles resolved by the cones. It begins to break down at the lowest cone resolutions because the geometrical images here begin to be wider than one cone. But at these low intensities the function is taken over by the rods, and the situation becomes quite different.

It is known anatomically for the central, foveal cones that they have a one to one connection with optic nerve fibers. This is borne out by the maximal resolution of gratings which corresponds precisely to the dimensions of single cones (Shlaer, 1937). For the rods, however, it is established that many elements are connected with a single nerve fiber, and this corresponds to their very low resolving power. In other words the unit of receptor action cannot be a single rod, but must be many rods. How many, one cannot say, but judging by the relative numbers of rods and nerve fibers it is certainly larger than ten, and may be a hundred or even more, though there is no reason to suppose the number of rods in a receptor unit to be the same for the whole retina. For our present purpose the precise number does not matter so long as it is more than a few rods. For convenience assume the number as five in linear dimension. Since the rods are about the same size as the central cones (Østerberg, 1935), the whole of the diffracted image of the wire will fall on this rod unit, and will be recognized only as a total decrease in the light. Any change in the depth and width of the shadow produced by the diffracted image of the wire results only in a decrease in the total light falling on the rod receptor unit, and this decrease will be directly proportional to the angular width of the wire since this determines the total amount of light removed. In short, here too the fractional intensity difference  $\Delta I/I$  will be directly proportional to the angular width of the wire. As for the cones, so for the rods provided that the

limiting factor in resolution is the retinal intensity discrimination, then the relation between visual angle and intensity should be the same as the relation between  $\Delta I/I$  and intensity

It is fortunate that the relation between the light intensity and the just perceptible fractional difference in intensity is known not only for the human eye, but for several other organisms as well. Moreover all the critical data for all animals have the same form, and can be described with high precision by the same type of equation (see the review by Hecht, 1937a). For the human eye the equation is

$$\Delta I/I = c[1 + 1/(KI)^{1/3}]^3 \quad (1)$$

where  $I$  is the prevailing light intensity,  $\Delta I$  is the just perceptible increment or decrement in it, and  $c$  and  $K$  are two constants of which  $c$  is the minimum value of  $\Delta I/I$  obtained at the highest intensities, and  $K$  is the reciprocal of the intensity at which  $\Delta I/I$  has a value of 4 times the minimum. The two constants have different values for cone vision and for rod vision, but the form of the equation is the same for both.

We have just seen that as a first approximation the critical visual angle  $\alpha$  for the resolution of a wire is directly proportional to  $\Delta I/I$ , that is  $\alpha = b'\Delta I/I$ . When this value is substituted in equation (1) it yields

$$\alpha = b[1 + 1/(KI)^{1/3}]^3 \quad (2)$$

where  $b = cb'$ . If equation (2) is plotted as  $\log \alpha$  against  $\log I$ , its form is invariant and independent of the numerical values of  $b$  and  $K$ . It is this equation (2) which is actually drawn through all the data in Fig. 2 both for the rod sections and for the cone sections. The value of  $b$  merely determines the position of the curve on the ordinates, while that of  $K$  fixes it on the abscissas. It is apparent that the equation describes both parts of the data with reasonable fidelity, and this may be taken as evidence for the idea that at any intensity the visual resolution of a single line is determined by the power for intensity discrimination of the retina at that light intensity.<sup>1</sup>

<sup>1</sup> In comparing measurements of visual function over a wide range of light intensities with such theoretical curves as given by equation (2) it is necessary to correct for varying pupil diameter (Reeves, 1918) and for pupil efficiency (Stiles

## VIII

*Visual Acuity and Illumination*

Our original purpose in using a single line was to enable us to describe in almost complete detail the light distribution on the retina. This has resulted in the present generalization about the interrelation between intensity discrimination and visual resolution at all intensities. May it not be that the same interrelation holds not only for the single line but for more complicated test objects as well?

It is certain that for at least one test object the interrelation cannot be carried over in its direct form. This is for the case of a repeating pattern such as a grating, because many factors besides intensity discrimination enter into the measurements. For instance, the maximum grating resolution can be sharply set by the dimensions of the receptor mosaic. This is true for the bee's eye (Hecht and Wolf, 1929), it also holds for the human eye (Shlaer, 1937) even when the intensity differences on the retina are quite large. Moreover in the human eye, pupil size can limit grating resolution not as it affects diffraction and intensity distribution but as it limits the transmission of the diffraction spectrum produced by the grating (Abbe, 1873, Shlaer, 1937). In other words, a grating, by its nature as a repeated pattern introduces extraneous elements into the problem.

Other test objects like a broken circle or a hook, though more complicated than one line, are nevertheless like it in being single units, and it is hard to see a really basic difference between such test objects and a line. The broken circle or the hook must suffer the same transformation of geometrical image as the wire, it is merely that the exact computation of the intensity distribution becomes very difficult indeed, so that one cannot envisage its precise relation to the retinal mosaic.

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and Crawford, 1933). Examples are Blanchard's data of instantaneous threshold (Hecht, 1937 b) and Koenig's data of visual acuity (Hecht, 1937 a). We have made such corrections in the present data, and find that because of the comparatively narrow range of intensities covered by each segment of the data, the pupil corrections do not change significantly the relation of the points in each segment to one another. As a result the theoretical curve fits the data either corrected or uncorrected for pupil

A significant point in this connection is that when the relation between visual acuity and light intensity is measured even with the common single test objects, the data also follow equation (2). This is true of Koenig's (1897) classical data using a hook as test object, which have been recomputed and replotted (Hecht, 1937*a*) and of Shlaer's (1937) very refined and critical data using a broken circle. Note that visual acuity is generally defined as the reciprocal of the just resolvable angle  $\alpha$  in minutes of arc subtended by the critical element in the test object, and that equation (2) may be plotted in its stationary state form as done by Shlaer. This agreement of visual acuity data with intensity discrimination data cannot obviously be considered as proof of their identity, the agreement, however, is pointed, when considered in the light of theoretical expectation.

#### SUMMARY

The visual resolution of a single opaque line against an evenly illuminated background has been studied over a large range of background brightness. It was found that the visual angle occupied by the thickness of the line when it is just resolved varies from about 10 minutes at the lowest illuminations to 0.5 second at the highest illuminations, a range of 1200 to 1.

The relation between background brightness and just resolvable visual angle shows two sections similar to those found in other visual functions, the data at low light intensities represent rod vision while those at the higher intensities represent cone vision. With violet light instead of white the two sections become even more clearly defined and separated.

The retinal image produced by the finest perceptible line at the highest brightness is not a sharp narrow shadow, but a thin broad shadow whose density distribution is described in terms of diffraction optics. The line of foveal cones occupying the center of this shadow suffers a decrease in the light intensity by very nearly 1 per cent in comparison either with the general retinal illumination or with that on the row of cones to either side of the central row. Since this percentage difference is near the limit of intensity discrimination by the retina, its retinal recognition is probably the limiting factor in the visual resolution of the line.



The resolution of a line at any light intensity may also be limited by the just recognizable intensity difference, because this percentage difference varies with the prevailing light intensity. As evidence for this it is found that the just resolvable visual angle varies with the light intensity in the same way that the power of intensity discrimination of the eye varies with light intensity.

It is possible that visual resolution of test objects like hooks and broken circles is determined by the recognition of intensity differences in their diffracted images, since the way in which their resolution varies with the light intensity is similar to the relation between intensity discrimination and light intensity.

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# THE CHLOROPHYLL-CARBON DIOXIDE RATIO DURING PHOTOSYNTHESIS

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The purpose of this report is to present experimental evidence indicating the formation—during the actual process of photosynthesis and not before—of a material in relatively large quantities which combines with or absorbs carbon dioxide. This material or substance will for the present be called “carbon dioxide combining intermediate” and certain of its definitely established properties or attributes will be described in this paper. The experiments establishing the existence of this carbon dioxide-combining intermediate are entirely new in type. Likewise the intermediate bears no evident relationship with any plant material previously described as combining with carbon dioxide, being produced, as it is, only during the actual process of photosynthesis. Its presence during photosynthesis can be measured by a “pick up” of carbon dioxide in darkness observable immediately following a high rate of photosynthesis. That the intermediate is chlorophyllous in nature is suggested by a simple stoichiometry of the order of unity that is found to exist between the number of carbon dioxide molecules taken up and the total number of chlorophyll molecules present in the plant. The importance of this newly observed carbon dioxide combining intermediate for our understanding of the process of photosynthesis as a whole is readily evident.

A simple chlorophyll carbon dioxide relation of some sort has been assumed in several theories of photosynthesis, although direct chemical evidence of any stoichiometric combination has heretofore been lacking. Emerson and Arnold (1932) demonstrated a linear relationship between chlorophyll content and maximum rate of photosynthesis in flashing light for *Chlorella*, but here some 2000 chlorophyll molecules seemed to be associated with the reduction of 1 carbon

dioxide molecule An uptake of carbon dioxide by dead leaf material was found by Willstatter and Stoll (1918) and by Spoehr and McGee (1924) that was suggestive of the formation of a complex, but it is questionable whether this uptake could be related to photosynthetic processes taking place in the living plant since it was found to occur in the material of only a few of many plants studied Willstatter (1918) has shown that carbon dioxide will form a compound with extracted chlorophyll only when the latter is subjected to a relatively high pressure of carbon dioxide gas

A detailed, and in the writer's opinion closely related, study of the induction phase is also presented in this report Here a certain simple stoichiometry between chlorophyll and carbon dioxide is observed which likewise provides evidence for the view to be supported in this paper that chlorophyll participates in the process of photosynthesis as an individual molecule rather than by units of several hundreds or thousands of molecules

The literature on chlorophyll photosynthesis contains few observations on the induction period, on namely, the time course of photosynthesis from the moment of illumination of a plant to the attainment of a constant rate of carbon dioxide assimilation or oxygen production under a given set of conditions The well known experiments of Osterhout and Haas (1918) are among the first recorded observations on the induction period Warburg's (1920) measurements of this phenomenon were made with his manometric method wherein instrumental time lag was overcome by integrating the effect of a series of light and dark periods In *Chlorella* he found a 2 minute induction period following a 10 minute rest in darkness Van der Paauw (1932) used about the same procedure with *Hormidium* and obtained similar results In addition he showed that a lower temperature lengthened the induction period The observations of Kautsky (1936) and of Franck and Wood (1936) on the fluorescence of chlorophyll in leaves of higher plants show a similar induction period with respect to fluorescence The earlier observations of the present writer (1937) were made with a spectrographic method of carbon dioxide measurement having so small a time lag that the integrational procedure was unnecessary These direct measurements of the time course of photosynthesis showed that essentially the same

induction phase in carbon dioxide assimilation—following a 10 minute “dark rest” (the period of darkness previous to the observation of the induction period)—occurred in a higher plant as in the algae studied by Warburg and Van der Paauw, and showed a greatly lengthened induction period following several hours of darkness. Smith’s (1937) manometric observations on *Cabomba* show an induction period of 4 or 5 minutes following more than 30 minutes darkness except for low light intensity where the period is about one half as long. Brigg’s (1933) observations on the non stomatal moss *Mnium* show an induction period of 25 to 30 minutes following more than 2 hours darkness. He states that “periods shorter than an hour seemed to give a less marked induction.”

The previous experiments of the author (1937), besides showing that practically the same induction phase occurred in the higher plant, wheat, as in the algae studied by previous workers, gave other evidence indicating that the mechanism for carbon dioxide assimilation is essentially alike in this higher plant as in algae, as might well be supposed. In studying the effect of intermittent illumination (equal light and dark periods) on carbon dioxide assimilation, a limiting increase in efficiency (per unit of light) of 100 per cent was approached as the length of the periods was decreased. At 1/60th of a second (the shortest used) the increase was about 90 per cent at 20°C, indicating essentially the same time factor for the Blackman reaction in the higher plant, wheat, as was found for *Chlorella* by Warburg (1919), and by Emerson and Arnold (1932).

In all experiments reported in this paper the same kind of plant, wheat (variety *Marquis*),<sup>1</sup> and the same spectrographic method of carbon dioxide measurement were used as formerly (1937). Assimilation measurements were taken at intervals of 30 seconds. The assimilation rates indicated graphically in the figures are the observed “apparent” rates and are uncorrected for respiration. All rates reported numerically in either text or figure refer to corrected net assimilation. In order to avoid ambiguity, the following terms are used in a specific sense throughout this paper: induction period, induction loss, dark rest, dark pick up, and plant uptake.

<sup>1</sup> Seed kindly supplied by Dr H. H. McKinney of the United States Department of Agriculture.

*A Dark Pick-Up of Carbon Dioxide by Green Leaves*

The following experiments were made primarily to compare induction under both light-limiting and carbon dioxide-limiting conditions at rates of assimilation higher than possible in normal air carbon

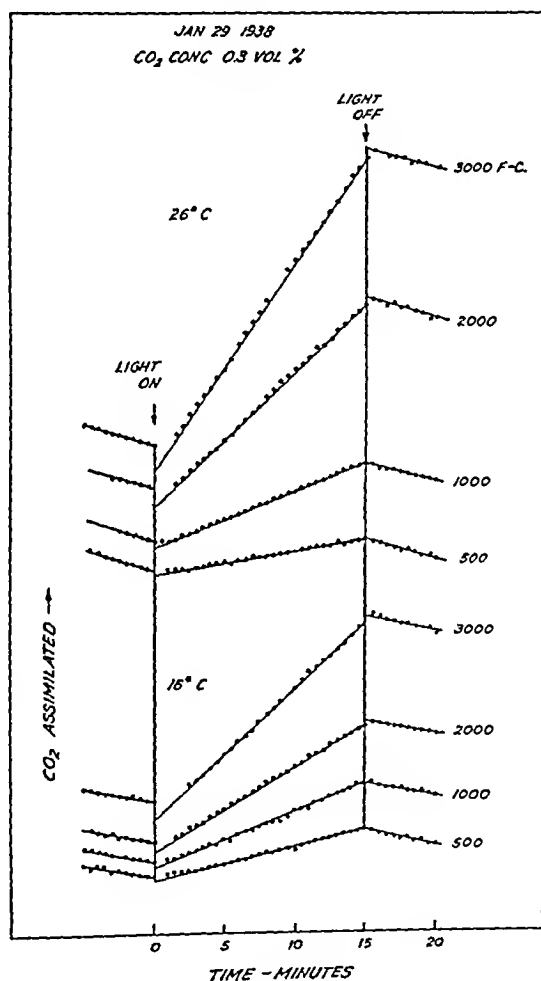


FIG 1 Time course of carbon dioxide assimilation at 16° and 26°C for four light intensities. The dark pick-up appears at the two higher light intensities.

concentration. However, they show the experimental conditions necessary for the observation of a dark pick-up of carbon dioxide so clearly that this more important matter will be taken up first. Employing a 10 minute dark rest throughout, the course of

assimilation was observed first under the conditions of normal air carbon dioxide (0.03 per cent), light intensity 3000 foot candles, and a temperature of 16°C. Then the carbon dioxide concentration was raised to 0.30 per cent and the course of assimilation observed for light intensities of 3000, 2000, 1000, and 500 foot candles. The entire experiment was then repeated at 26°C. In each separate experi-

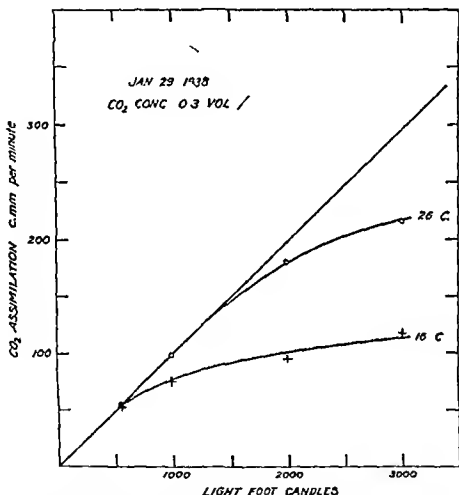


FIG 2 Rate of carbon dioxide assimilation *versus* light intensity (from Fig 1) showing the nature and extent of the various limiting factors

ment the plant was exposed to the chosen light intensity for about 10 minutes prior to the 10 minute dark rest. The data obtained for the higher carbon dioxide concentration are shown in Fig 1. Here the ordinate is carbon dioxide assimilated and the abscissa is time. The downward slope before and after the period of illumination (15 minutes) of the plant gives the rate of respiration. From these data, Fig 2 has been drawn to indicate better the nature and extent



of the limiting factors concerned in the various curves in Fig 1 It is of interest to note here in passing the usual approach to a zero temperature coefficient under light-limiting conditions

It is immediately apparent when Fig 1 is examined that at the two

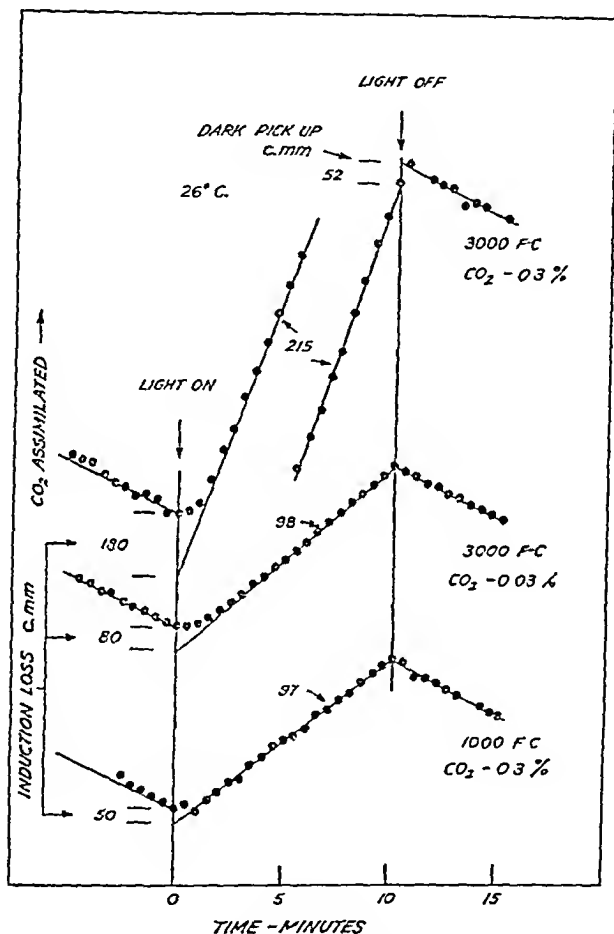


FIG 3 Time course of carbon dioxide assimilation at 26°C under different light and carbon dioxide conditions

higher light intensities the carbon dioxide assimilation continues for about 20 seconds *after the plant is darkened*, indicating a dark pick-up of carbon dioxide immediately following a relatively high rate of photosynthesis. The upper curve in Fig 3 shows this pick-up of carbon dioxide in darkness (and the conditions under which it is

observed) more clearly than the several curves of Fig 1, because of the larger scale employed. In Fig 3 the lower curve shows the course of assimilation under the conditions of carbon dioxide concentration 0.3 per cent and light intensity 1000 foot candles. The middle curve shows the course of assimilation under the conditions of carbon dioxide concentration 0.03 per cent and light intensity 3000 foot-

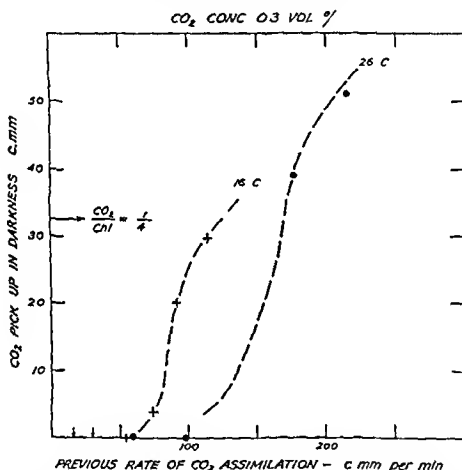


FIG 4 Relationship of the dark pick up of carbon dioxide to the previous rate of photosynthesis (These data might better be represented by a single S shaped curve)

candles. These two sets of conditions balance to give in both curves about the same rate of assimilation (corrected for respiration)—97 and 98 c mm per minute (at 20°C and 760 mm pressure), but a scarcely appreciable pick up either under light limiting or carbon dioxide limiting conditions where the rate is greatly lowered. The experimental error here is about  $\pm 5$  c mm. It should be noted that the greater smoothness of the data for the middle curve in Fig 3

is due to the greater sensitivity of the spectrographic method at this lower carbon dioxide concentration. The upper (broken) curve in Fig 3, where a much higher rate of assimilation (215 c mm per minute) was obtained, shows the nature and extent of the observed dark pick-up. Here it is seen that the carbon dioxide assimilation continues for about 20 seconds after cessation of illumination, so that the line for the respiration rate drawn back to the time of light cessation shows an intercept giving the amount of the pick-up in darkness (52 c mm, upper curve).

In Fig 4 the dark pick-up is plotted (data from Fig 1) against previous rate of carbon dioxide assimilation as governed by varying light intensity. From these curves it follows that the dark pick-up is observable experimentally *only* following a relatively high rate of assimilation. Fig 4 indicates little or no pick-up observable at low previous rates of assimilation, but as the rate is increased by increasing light intensity the observable pick-up becomes marked. It is possible that a single curve would better represent the data in Fig 4. The compensation points (where respiration balances assimilation) for 16° and 26°C (17 and 32 c mm / min) are indicated on the abscissal scale by small arrows. In Fig 1 it is seen that in both the 3000 foot-candles experiment of the 16°C group and the 2000 foot-candles experiment of the 26°C group a marked pick-up is apparent. The latter case is under practically light-limiting conditions while the former is under strong carbon dioxide limitation (see Fig 2). It appears then that high previous rate of assimilation (necessarily high light) rather than any particular limitation of carbon dioxide or light is essential to the observation of the pick-up.

Further experiments were made to determine the relation between time of illumination and appearance of the pick-up. Here the carbon dioxide concentration used was 0.30 per cent, the temperature 21°C, and the light intensity 4000 foot-candles. After a dark rest of 10 minutes in each case the course of assimilation was observed for a light period of 5 minutes, 2 minutes, 1 minute, and 30 seconds. Typical results are shown in Fig 5. It is apparent here that the full pick-up is attained by the time the plant is out of the induction period. The inset in Fig 5 compares the rate of assimilation during and after the induction period with the corresponding pick-up observed. The

ordinate scale for the pick up has been adjusted to bring the maximum pick up and equilibrium rate of assimilation together. Here it is evident that the pick up is closely correlated with the rate of assimilation during the induction period.

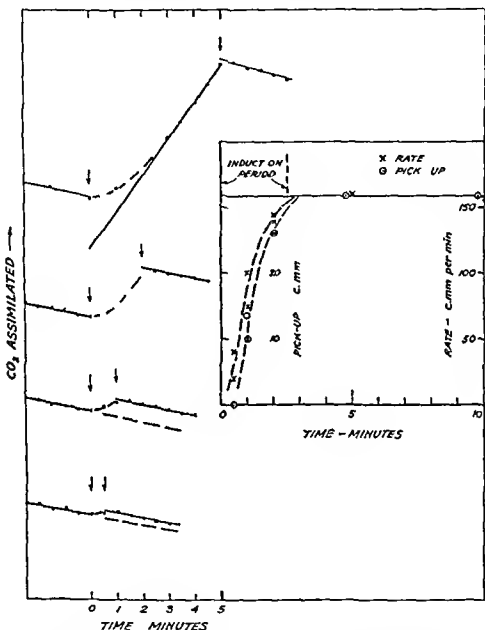


FIG 5 Dark pick up *versus* time of illumination, showing that the maximum pick up is attained as soon as the plant is out of the induction period

lation during the induction period. The experimental error in measuring a pick up for 30 seconds and 1 minute illuminations is large. Values of the pick up plotted in the inset for these times were obtained by comparing the vertical difference between the straight

lines representing the respirational rate before and after the light periods with corresponding ordinates (mean values) on the 5 minute and 2 minute curves. A small pick-up was indicated for the 1 minute period and essentially none for the 30 second period. The experiments shown in Fig 5 indicate, interestingly enough, that an inherent error is present in rate measurements during the induction period when the manometric integrational procedures heretofore used (as by Warburg (1919)) are followed, for the pick-up is included in the measurement for each time interval, producing thereby an apparent rate higher than the actual "instantaneous" rate.

That the pick-up of carbon dioxide in darkness is not an instrumental effect is evidenced by the fact that the instrumental lag is about 3 seconds, whereas the effect corresponds to about a 20 second lag. The data shown in Fig 4 are uncorrected for this lag. For a previous rate of 100 c mm per minute the true pick-up is no more than 5 c mm less than shown.

Many different tests were made to verify the experimental reality of the pick-up of carbon dioxide in darkness described. In every case where the previous rate of carbon dioxide assimilation was several times that possible in normal air carbon dioxide concentrations, a definite pick-up was observed. In one typical experiment where the light intensity was 8000 foot-candles, carbon dioxide concentration 0.15 per cent, and rate of carbon dioxide assimilation 240 c mm /min, the pick-up in darkness was 40 c mm. Comparing this with the data shown in Fig 4, it is apparent that in these experiments the previous rate of carbon dioxide assimilation was the main factor determining the pick-up of carbon dioxide. It is of interest to mention in passing that the maximum rate of 215 c mm per minute (Fig 2) is about three times the maximum value per unit wet weight observed by Smith (1937) with *Cabomba* at three times the present carbon dioxide concentration and ten times the present light intensity. These high assimilation values of wheat are still somewhat below those reported by Alten and Goeze (1938).

Further experiments were made in an effort to determine if this pick-up might possibly be due to "free" chlorophyll. In these experiments the plant was "conditioned" in carbon dioxide-free air and high light intensity for one-half hour, this treatment supposedly free-

ing chlorophyll Carbon dioxide was then suddenly (2 seconds) admitted to the system without break in illumination, and the subsequent course of assimilation in light observed (In some experiments the plant was darkened when the carbon dioxide was admitted and the subsequent course of respiration was observed) In these tests when only enough (exactly measured) carbon dioxide was added to bring the concentration to the normal air value of 0.03 per cent, no appreciable pick up of carbon dioxide was observed (the small amount needed to saturate the plant volume is less than experimental error) When more carbon dioxide was added—for example, enough to bring the concentration to 0.3 per cent—the experimental error in delivering the 6000 c. mm. of carbon dioxide needed was of the same order (60 c. mm.) as the best pick up yet observed Probably the most critical experiment (least experimental error) showing no pick-up after these particular conditioning periods was when no carbon dioxide was added subsequent to conditioning Here the system was simply closed on zero carbon dioxide and the subsequent course of respiration in darkness or assimilation (of respired carbon dioxide) in light observed An example of the latter will be shown in Fig. 9 In these experiments there was no decrease in rate of respiration when the system was closed and the plant darkened, and no significant departure from a linear relationship between rate of assimilation and carbon dioxide concentration when the light was left on the plant after the system was closed

#### *The Relation of the Induction Phase to the Previous Dark Rest*

It was found earlier by the writer (1937) that the induction period was many times longer after a night (10 hours) of darkness than after a short (10 minute) dark rest This fact led the writer to study this phenomenon for a range of dark rests from 20 seconds to several hours The results of these observations are shown in Fig. 6 Here the carbon dioxide concentration was that of normal air (0.03 per cent), the light intensity was 1500 foot candles, and the temperature 20°C A study of Fig. 6 will show that both the length of the induction period and the amount of carbon dioxide involved ("induction loss") are determined by the previous dark rest

The marked dependence of the length of the induction period upon

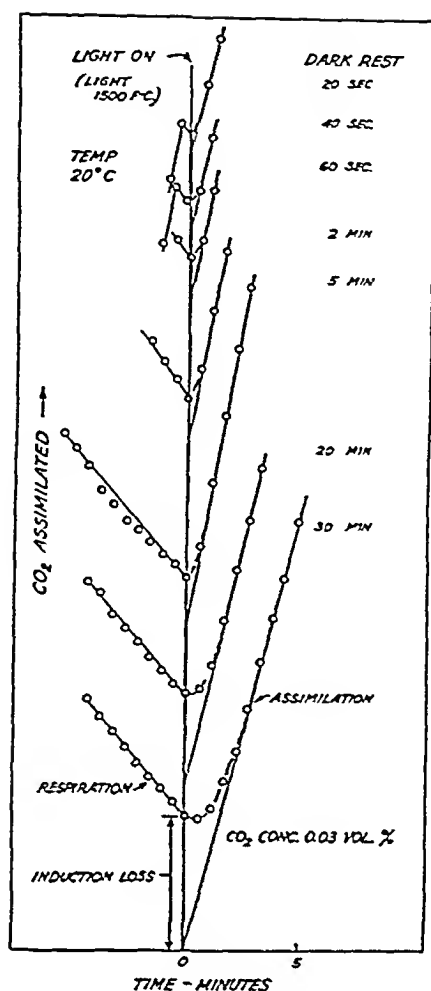


FIG 6 Time course of carbon dioxide assimilation for different previous dark rests, showing the effect of this dark rest upon the induction period and induction loss

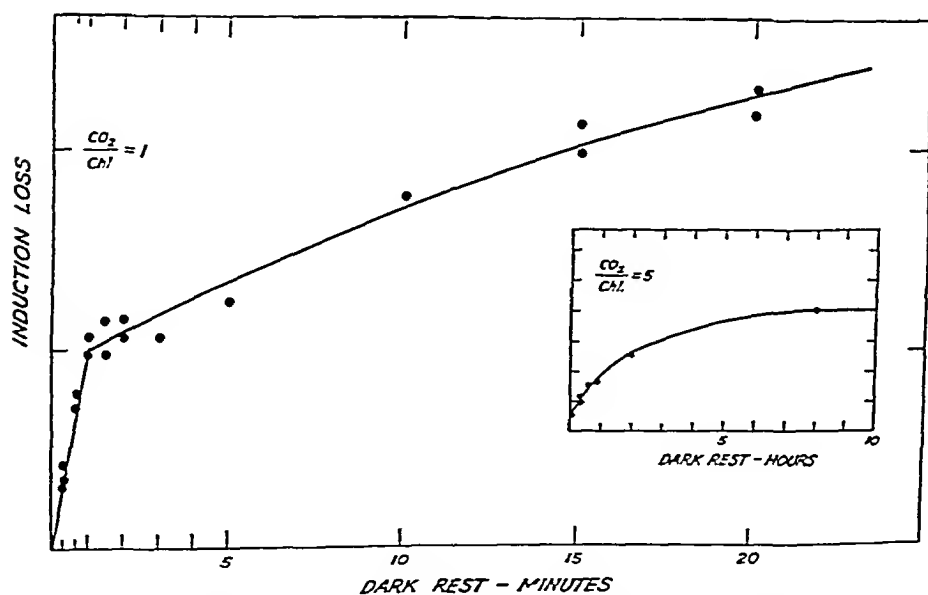


FIG 7 Induction loss versus dark rest, showing the time course of the reactions occurring in the dark which are responsible for the phenomenon of induction

the previous dark rest shown in Fig 6—thus for a 30 minute dark rest it is two to three times as long as for a 5 minute dark rest and after a night of darkness it is 15 minutes or more—tends to bring together the rather discordant values reported in the literature previously mentioned. It is evident now that the main factor operating to give the range of values found in the literature has been the length of the dark rest employed. This is more likely than the suggestion of Manning (1938) that the diversity of characteristics of the induction period for different plants may be due to a different mechanism for induction in different plants, and is quite in line with the view expressed by Burk and Lineweaver (1935) that “with a process so highly specific, it is unlikely that nature would develop a wide variety of fundamentally different paths”. It was pointed out by McAlister (1937) that the ordinate interval between any given curve at the point of illumination (light on) and the intersection of the straight line of the constant final rate of assimilation extended backward to this time represents an amount of “carbon dioxide lost to photosynthesis” during the induction period, an amount hereinafter to be called induction loss. The induction loss, which is both a different and a more quantitative measurement of induction than the length of the induction period, is plotted on the ordinate axis against the corresponding dark rest on the abscissal axis in Fig 7. (The ordinate scale actually used is the molar ratio of induction loss to chlorophyll content.)

The data for Figs 6 and 7 were obtained in the following manner. The plant was given a certain period of darkness and then the course of assimilation observed at 30 second intervals, giving a curve in Fig 6. The induction loss for this dark rest gives one point on Fig 7. This was repeated for all the dark rests shown, the experiments not being made in order, until enough data were obtained to establish the shape of the curves shown in Fig 7. In this way, *i.e.* by studying induction after different dark rests, the time course of the dark reaction or reactions responsible for the induction phase was observed. Fig 7 actually gives evidence of *two different dark reactions* being involved. The first is a rapid reaction progressing approximately linearly with dark time (zero order) and attaining completion in about 1 minute (at 20°C). The second is a much slower reaction that goes on for a number of hours (see especially inset).



The relation of the induction loss to the amount of chlorophyll present in the plant was obtained by measuring the chlorophyll content of the plants (the blades and sheaths of the leaves only were assayed) at the end of a 24 hour series of observations, yielding the results indicated in Fig 7. The chlorophyll content was estimated by the method of Willstätter and Stoll, as modified by Schertz (1928), comparison being made spectrophotometrically with a standard sample of chlorophyll obtained from Dr Schertz. An average value of 1.06 per cent of the dry weight of the leaves for total chlorophyll was obtained. The ratio of the number of moles of carbon dioxide in the induction loss to the number of moles of chlorophyll present in the leaves of the plants under test is used as the ordinate scale in Fig 7. By assuming that van den Honert's (1930) assimilation number for *Hormidium* applies to van der Paauw's data (1932) on induction with *Hormidium*, the induction loss may be compared with chlorophyll content in the latter experiments. An estimated ratio of about unity is obtained. Calculation of Smith's (1937) data, with the assumption of the same chlorophyll content per unit wet weight as in wheat, also yields a ratio of the order of unity.

Fig 7 agrees very well with Fig 4 in the paper by Franck and Wood (1936). These authors were observing curves of recovery of fluorescence in green leaves in darkness, and they state "They [the curves] are strictly linear. It seems that after the apparent equilibrium is reached (which takes the time of the order of magnitude of one minute) a very slow and small rise takes place for hours, as is indicated by the higher maximum observed after a whole night's repose in the dark." These two entirely different methods of observation have thus yielded almost identical results, and the close relationship between fluorescence of chlorophyll and photosynthetic induction is almost certainly established.

The experiments of Fig 6 were all made with normal air carbon dioxide concentration and a light intensity high enough for carbon dioxide to be somewhat limiting. In order to compare induction under both light-limiting and carbon dioxide-limiting conditions, at higher rates of assimilation, the previously described experiments shown in Figs 1 and 2 were made. When Fig 1 is examined, keeping Fig 2 in mind, it is apparent that in passing from light-limiting to

carbon dioxide limiting conditions (here low to high light), all else being constant, there is a marked increase in the amount of carbon dioxide lost to photosynthesis during induction (induction loss, see Fig 6)

The rather abrupt increase in induction loss as carbon dioxide becomes limiting shows up more clearly in Fig 8, where the induction losses observed in Fig 1 are plotted against the rate of assimilation attained For both 16° and 26°C, the loss goes above the extended straight line that holds for light limiting conditions (In regard to how far this "light limited" line would continue linear—with either the 10 minute dark rests here employed or other durations—it is well to bear in mind that the abscissa is not light intensity but rate of assimilation as determined by light intensity) Smith's (1937) data on induction when similarly treated yield a curve like those in Fig 8 The induction losses for the normal air case (0.03 per cent carbon dioxide) at 16° and 26°C (shown here by two particular points only) also appear above this straight line, again indicating somewhat more carbon dioxide lost to photosynthesis, for a given rate of assimilation, under carbon dioxide limiting than under light limiting conditions This comparison is made even more exactly and strikingly in the previously described experiments shown in Fig 3 Here the two sets of conditions for the two lower curves balance to give about the same rate of assimilation (corrected for respiration)—97 and 98 c mm per minute—but the induction loss is considerably greater under carbon dioxide limiting conditions (80 c mm) than under light limiting conditions (50 c mm) The experimental error here is at most  $\pm 5$  c mm The upper (broken) curve in Fig 3, where carbon dioxide is the main limiting factor at a higher rate of assimilation (215 c mm per minute), shows an even greater induction loss (180 c mm)

The results shown in Figs 3 and 8 indicate that the induction losses given in Fig 7 would be reduced by about one half if this experiment had been performed under light limiting conditions, although this cannot yet be definitely stated except for the 10 minute dark rest period, complete data not being at hand

Experiments that need not be discussed here have established another fact of interest, namely An induction period is observed in

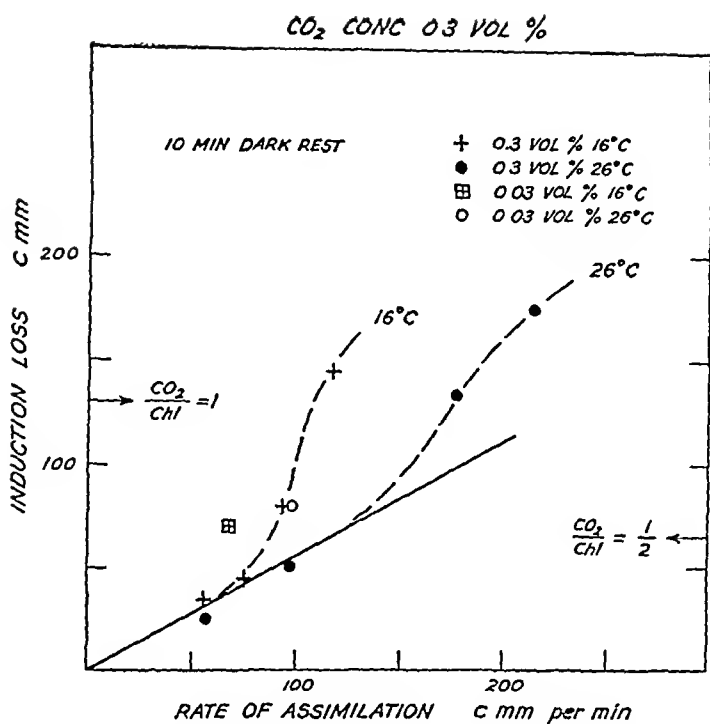


FIG 8 Induction loss *versus* rate of assimilation, showing a linear relationship under light-limiting conditions

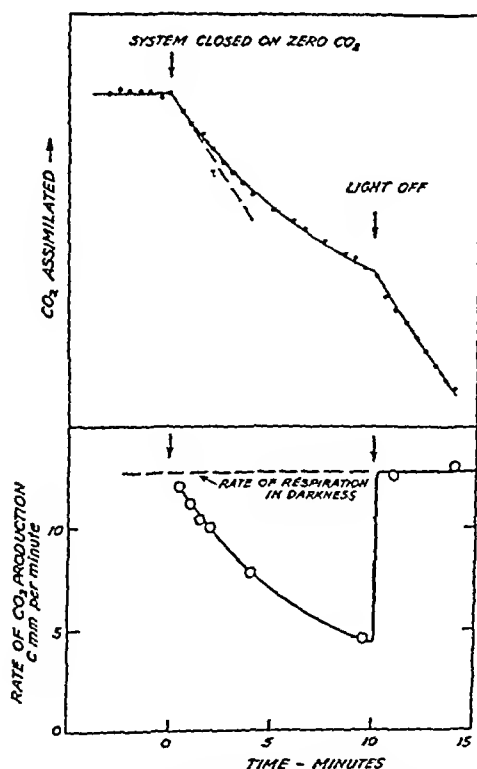


FIG 9 Time course of carbon dioxide production after a conditioning period (see text), showing no pick-up after conditioning and no effect of light on respiration

passing suddenly from a long illumination at low light intensity to high light intensity. The induction losses found in changing from darkness to low light and from low light to high light add up to approximately the same induction loss as is found in passing from darkness to high light. The converse of this experiment is yet to be performed and will have especial interest in relation to the dark pick up, which has so far only been measured upon passing to zero rather than lowered light intensities.

In the experiments where carbon dioxide was admitted after the conditioning period it was quite clear that the plant was brought out of the induction phase by the conditioning in light even in the absence of carbon dioxide. Here no induction period in the rate of assimilation was apparent when the carbon dioxide was suddenly admitted to the plant chamber after several minutes of intense illumination. The assimilation began immediately at about the otherwise normal value. In other words the presence of carbon dioxide (in measurable concentration) is not necessary to bring the plant through the induction period upon exposure to light.

#### *The Absence of a Direct Effect of Light on Respiration*

In some of the conditioning experiments previously mentioned is found unmistakable evidence that light has no effect on respiration in the higher plant used. This was an unplanned result, but is of such pertinent interest as to call for mention here. One of these experiments is shown in Fig. 9. Here, before the system was closed, air free from carbon dioxide but with normal oxygen content was rapidly blown through the plant chamber effectively keeping the plant under carbon dioxide free conditions. This treatment was continued for one half hour. The light intensity on the plant was 4000 foot-candles and was maintained when the system was closed. The upper curve in Fig. 9 shows, first at the left, readings at a constant level for no carbon dioxide in the system, then at time marked zero the system was closed and respiration appears immediately at a rate equal to the dark value even though the plant was illuminated. Very soon, however, some carbon dioxide concentration is built up and assimilation sets in as seen by the departure from the dashed line representing respiration and by the slowly reduced rate of production of carbon dioxide shown in the lower curve. After 10 minutes of this the plant

was darkened and the dark respiration appears immediately at the same rate as when the plant was illuminated in the absence of carbon dioxide. If any effect such as reported by van der Paauw (1932) was present in these experiments the rate of production of carbon dioxide when the system was first closed would have been more than twice the dark rate shown, rather than unchanged. This result is in agreement with previous but less direct experiments of the writer (1937).<sup>2</sup>

#### DISCUSSION

Before any significance for photosynthesis can be attached to the pick-up of carbon dioxide in darkness, it is desirable to ascertain if this amount is greater than that which the plant volume (independent of any specific intermediate binding) would be expected to take up in bringing itself to equilibrium with the carbon dioxide concentration in the surrounding air, *i e*, 0.3 per cent. The volume of leaf material in the plant used in the experiments of January 29, 1938, was 2.7 cc (*i e*, wet weight 2.73 gm). The maximum possible plant uptake to satisfy a deficit under the most extreme carbon dioxide-deficient conditions would be the amount necessary to bring the total carbon dioxide concentration in the plant from 0 to 0.3 per cent, which (assuming an absorption coefficient of about unity as for water<sup>3</sup>), is  $2.7 \text{ cc} \times 0.003 \text{ atmospheres} \times 1$ , or about 8 c mm. This is less than one-sixth the amount observed (see Fig. 4), and much less than one-sixth assuming less than extreme carbon dioxide limitation. It is also improbable that a buffer solution in the plant would be exhausted during photosynthesis and should be filled up in the dark because this would take more than 20 seconds time. Consequently it is believed that the observed dark pick-up has a real bearing on the mechanism of photosynthesis. It is of interest to note that if the experiment were made at 3 per cent carbon dioxide (higher concentration) and (of course) higher light, the pick-up obtained, if not too greatly different than already measured, would be of about the same amount as that due to the plant uptake, *i e* for 3 per cent carbon dioxide concentration the maximum plant volume uptake would be about 80 c mm. Thus the dark pick-up is probably most clearly observable

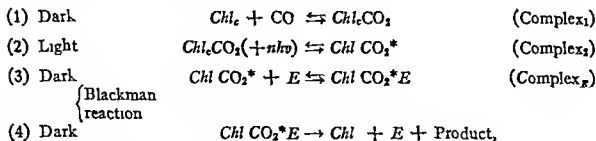
<sup>2</sup> This is contrary to the recent suggestion of Blinks and Skow (1938 *b*)

<sup>3</sup> See Smithsonian Physical Tables, Washington, D. C., Smithsonian Institution, 8th edition, 1933, 221

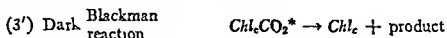
at some intermediate carbon dioxide concentration, of the order actually used, rather than at some extreme concentration. This reasoning presupposes, of course, that at much higher assimilation rates than yet achieved a limiting pick up set by the number of intermediate molecules would be obtained (even if not yet observed), increasing carbon dioxide concentration would thus continue to increase the plant uptake in relation to dark pick up and increasingly obscure the latter beyond a certain set of optimal values.

This brings us to the most important conclusion of the present paper, namely the facts that this dark pick up (1) is an experimental order of magnitude greater than that expected for the plant volume coming to equilibrium with its surroundings (plant uptake), (2) cannot be due to the action of a buffer solution in the plant, and (3) is of an amount—molecule for molecule—equal to one quarter or more of the total chlorophyll present (see Fig 4), strongly suggest the formation during photosynthesis of a simple carbon dioxide combining intermediate very probably chlorophyllous in nature. It is believed that a curve such as shown in Fig 4 represents roughly the functional relation between the "population" of the intermediate (or intermediates) and the rate of photosynthesis.

It is very interesting to note that the pick-up and its marked function of previous rate of assimilation are called for and theoretically predicted by the well known kinetic mechanism proposed by Burk and Lineweaver (and several others in less detail) (1935). Employing a minimum of one light and three dark reactions, the schema of Burk and Lineweaver (for the steady state rate of photosynthesis after the induction phase) is as follows



or where saturation of the Blackman enzyme *E* need not be considered, as ordinarily, (3) and (4) may be written



*Chl<sub>c</sub>* is a chlorophyllous intermediate or complex

This mechanism explains the observed pick-up of carbon dioxide in darkness as follows. The amount of the pick-up is determined by the amount of  $Chl_c$  involved in (3'). Darkening the plant stops (2), and (3') continues for a fraction of a second until all the chlorophyll complexes concerned therein become  $Chl_c$ . It is (1) that is responsible for the observed pick-up of carbon dioxide in darkness. Since the amount of  $Chl_c$  involved in (3') is a function of the rate of assimilation, the pick-up due to (1) is obviously a marked function of the previous rate of carbon dioxide assimilation. The concentration of Complex<sub>2</sub> does not increase linearly with rate, or conversely decreases faster than the rate falls as suggested by the shape of the curves in Fig. 4. The amount of carbon dioxide involved in any pick-up so far observed, *z e* the maximum ratio of carbon dioxide to chlorophyll, is as yet only about 1/4 (Fig. 4), but this is quantitatively as well as qualitatively expected by the mechanism of Burk and Lineweaver since in the experiments concerned the assimilation rate attained was still only of the rough order of 1/4 or less of the maximum possible if the carbon dioxide and also the light were completely nonlimiting. As already explained, to measure a pick-up ratio of nearly unity involves some special difficulties, and to obtain a ratio of 1/4 under the experimental conditions employed is equivalent to obtaining a ratio approximating unity (or even 2 in the event of  $Chl_c(CO_2)_2$ ) under conditions of saturating carbon dioxide concentration and light intensity.

The writer realizes that it is a problem as to what happens to the carbon dioxide picked up, if no light is added for a long time. A slow dissipation (zero order or otherwise) would be lost in respiration. A correlation of the probable disappearance of the intermediate (or intermediates) with the dark reactions shown in Fig. 7 suggests itself, but this conjecture is probably premature.

Further striking indications of a simple carbon dioxide-combining chlorophyllous intermediate, as postulated in the commonly conceived mechanisms collated by Burk and Lineweaver (1935), derive from the quantitative aspects of the time found to be involved for the dark pick-up, 10 to 20 seconds. The pick-up can scarcely be much faster than the previous rate of assimilation in view of the partial  $CO_2$  limitation operating. In Fig. 3, top curve, the assimi-

tion rate is 215 c mm per minute. The number of chlorophyll molecules, on a basis of a  $1/4$  to  $1/2$  pick-up, is equivalent to about 50 c mm of carbon dioxide. Hence 50/215 minutes or about 15 seconds is the order of time required. It might have been thought that a pick-up was bound to be involved at a high rate of assimilation under carbon dioxide limiting conditions, but the order of magnitude of time required, in relation to the previous rate of assimilation calls for a number of molecules of carbon dioxide predicted by the size of the chlorophyll unit, thus if the pick up corresponded to a unit of 2000 molecules of chlorophyll per molecule of carbon dioxide the time calculated would have been 15/2000 seconds, and not 15 seconds as on a basis of unity.

The experimental evidence so far at hand indicates that the material responsible for the pick up is an intermediate formed only during photosynthesis. If a pick up had been observed after the conditioning treatments used it would have suggested that ordinary free chlorophyll in the plant combined directly with carbon dioxide and that this free chlorophyll was responsible for the observed pick up. The conditioning experiments were planned with this possibility in mind, it being thought that strong illumination in the absence of carbon dioxide would, by the process of photosynthesis, free the chlorophyll from any carbon dioxide bound to it, so that when carbon dioxide was admitted after the conditioning it would immediately become attached to the chlorophyll. Since no pick up was observed here we must conclude either that an intermediate formed during photosynthesis is responsible for the observed pick up or, as there would be little reason to suppose, that the chlorophyll was not made free by the conditioning treatment.

The striking correlation between the progress of the fast dark reaction shown in Fig 7 and the recovery of fluorescence of chlorophyll in leaves in darkness shown by Franck and Wood (1936) is of importance in demonstrating a real relation of fluorescence to photosynthesis. This rapid dark reaction, going to completion in about 1 minute, may logically be associated with the minimum growth effects found in intermittent light experiments at intermittencies of about 1 minute by Garner and Allard (1931), Iggena (1938), and others.



The earlier studies of the author (1937) and the data herein reported on the induction phase bring out clearly the fundamental nature of this phenomenon. It has been shown that both the time factor and the induction losses are essentially the same in higher plants as in algae. The present studies of the induction loss have shown the same time factor and order (zero) for the dark reaction causing induction as was found by means of the fluorescence of chlorophyll (Franck and Wood (1936)). The linear relation between induction loss and attained rate of assimilation under light-limiting conditions also emphasizes a fundamental relation between induction and photosynthesis. In view of all this it appears reasonable to the writer to assume that the number of carbon dioxide molecules "lost to photosynthesis" during induction (the induction loss) gives an approximate measure of the number of elementary photosynthetic units or cycles unoperative or compensated for during induction. Some chlorophyll units are no doubt involved in the cycle more than once during the induction period, which complicates the "counting." Nevertheless, the fact that the molecular ratio of the induction loss (after 1 minute dark rest) to total chlorophyll is of the order of  $1/2$  leads the writer to believe that chlorophyll enters into the photosynthetic process as essentially an individual molecule, and not as part of a very large multimolecular unit. It must be admitted that this does not offer immediate help in explaining light saturation under high carbon dioxide concentrations. It does indicate, however, the necessity of an explanation of this along lines other than that of the assumption of a "photosynthetic unit" of the order of 2000 chlorophyll molecules associated with the reduction of one carbon dioxide molecule, some explanation for example, of the type offered by Franck and Herzfeld (1937).

#### SUMMARY

Using a rapid spectrographic method of carbon dioxide measurement previously described by McAlister (1937) further studies on the time course of photosynthesis in the higher plant, wheat, variety *Marquis*, are herein reported. Of major importance in this work is the discovery of a pick-up of carbon dioxide in darkness immediately

following a high rate of photosynthesis (see Figs 3 and 4). This pick up is believed to be due to the action of a carbon dioxide combining intermediate, *i.e.*, the "acceptor molecule" for carbon dioxide in photosynthesis. The conditions under which this phenomenon has so far been observed indicate that the intermediate is formed in relatively large quantities during the actual process of photosynthesis and not before. That the intermediate is chlorophyllous in nature is suggested by a simple stoichiometry of the order of unity that is found to exist between the number of carbon dioxide molecules taken up and the total number of chlorophyll molecules present in the plant. This is in opposition to the idea of a large photosynthetic unit of some 2000 chlorophyll molecules operating together in the reduction of 1 carbon dioxide molecule.

Further studies of the induction phase under various conditions of previous dark rest and of carbon dioxide and light limitation are herein described. Employing the simple hypothesis that the number of carbon dioxide molecules not reduced during the induction period (induction loss) gives a measure of the number of elementary photosynthetic cycles unoperative or compensated for during induction together with the experimental fact that this induction loss is of the order of the total number of chlorophyll molecules present, these latter studies also indicate, in a less direct manner, that chlorophyll participates in photosynthesis as an individual molecule and not as part of a very large multimolecular chlorophyll unit.

The fast dark reaction lasting about 1 minute (Fig. 7) required to reproduce both (a) the phenomena of induction in carbon dioxide assimilation and (b) the recovery of fluorescence of chlorophyll in leaves in darkness as observed by Franck and Wood (1936), demonstrates a close relationship between the fluorescence of chlorophyll and induction in photosynthesis.

The rate of respiration (carbon dioxide production) of the higher plant, wheat, was measured under intense illumination and in the absence of carbon dioxide (to suppress assimilation). This value was found to be identical with the dark respirational rate measured before and after the light period, indicating very positively the absence of any direct effect of light on respiration.

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# THE EFFECTS OF PANTOTHENIC ACID ON RESPIRATORY ACTIVITY\*

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## I

### INTRODUCTION

Pantothenic acid is known to be present in a wide variety of tissues (1, 2) Its ubiquity suggests that it is a factor essential to cell physiology rather than a compound with a more localized function Results have been published showing its effects on yeast growth (1) and indicating a relationship with the carbohydrate metabolism of both yeast and green plants (alfalfa) (3, 4) In the experiments here reported it has been found that pantothenic acid has stimulative effects on the respiration of yeasts, on fermentation by non living enzyme preparations, and on the respiration of certain vegetable tissues

All of the respiration studies were made with a fourteen unit Warburg Barcroft microrespirometer A temperature of 30°C and an atmosphere of air was used in all cases The two vessel method outlined by Dixon (5) was used with the following precautions (1) Shaking at a rate of 100 oscillations per minute and an amplitude of 4 cm eliminated any diffusion effects (2) Buffers (phosphate) were used to maintain constant pH (3) Manometers were opened frequently (between readings) so that the O<sub>2</sub> pressure was not significantly lowered (4) Whatman No 40 "KOH papers" were used and found to give accurate results even when the O<sub>2</sub> consumption was small (5) The pantothenic acid was added as the calcium salt

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in a preparation which was approximately 25 per cent pure. Much previous work on material of even lower purity indicated that the preparations are biologically pure. The calcium ion was proved not to be responsible for the effects by testing a range of concentrations of  $\text{CaCl}_2$ . This was probably an unnecessary precaution since extremely low concentrations of the pantothenic acid were used. (6) Proper thermobarometric and other controls were run.

## II

### *Effects on the Respiration and Growth of Living Yeasts*

In this part of the study the effects of pantothenic acid on yeast respiration and growth were investigated and its effects compared with, and related to the effects of certain compounds known to be biochemically important (6-8).

Two yeasts were studied. (1) Gebruder Mayer yeast grown on Williams' medium (9) plus a low dosage of pantothenic acid. This is a synthetic medium except for the pantothenic acid which must be added in order to get a crop large enough for the experiments. (2) Fleischmann's yeast used directly from the center of a fresh retail cake. This yeast affords a readily available standard and its respiration was found to be reasonably constant from day to day. The G M yeast when grown as described is deficient in all factors which it cannot synthesize. An assay of the pantothenic acid present indicated that it had less than one-tenth as much of the acid as normal yeast. Similar deficient G M yeast was used in the previously reported preliminary experiments on the effect of pantothenic acid on  $\text{CO}_2$  and  $\text{O}_2$  exchange (3).

Three basal synthetic media of increasing degrees of completeness were used. (1) 0.2 molar c.p. sucrose. (Experiments using ordinary sucrose indicated that it contains a respiration stimulant.) (2) Medium 1 plus the inorganic salts and "trace elements" of Williams' medium in twice the concentration in which they are found in that medium (9). (3) Medium 2 plus 10 gm *l*-asparagine per liter. As used in the respirometer flasks the final medium contained 1 cc of one of these three basal media, 1 cc of  $\text{KH}_2\text{PO}_4$  solution (4 gm per liter), and 0.4 cc of distilled  $\text{H}_2\text{O}$  containing the compound to be studied.

The following compounds were investigated Pantothenic acid, thiamin (Merck's Betabion),  $\beta$  alanine, ethanolamine, meso inositol, indole 3 acetic acid, nicotinic acid, pimelic acid, uracil, and 3,5 dinitro-o cresol Liver extract (Lilly's No 343), representing a crude tissue extract, was also studied Numerous experiments done with these materials over a range of concentrations showed that pantothenic acid, thiamin,  $\beta$  alanine, ethanolamine, and the liver extract gave, under the conditions used, definite stimulation, while no appreciable acceleration of the respiratory rate was obtained with any of the other compounds Since ethanolamine was effective only in relatively large concentrations, and since  $\beta$  alanine doubtless owed its effect to its intimate structural relation to pantothenic acid (10), it was decided that pantothenic acid, thiamin, and the liver extract should be more intensively investigated

In all experiments the yeast was washed once by centrifugation from  $\text{KH}_2\text{PO}_4$  solution and then re suspended in fresh phosphate solution at a concentration of approximately 4 mg per ml for pipetting into the flasks The actual concentration was determined by the thermocouple method (11) Parallel runs on 2, 4, and 6 mg of yeast proved that over this range the respiration rate (per milligram) did not vary materially with the weight of yeast present Respiration was usually measured over a period of about 2 hours and growth was determined by a second reading on the thermocouple after 6 hours shaking in the apparatus Although at high concentrations certain compounds produce osmotic effects which alter the thermocouple readings it was found that the small variations in concentration resulting from the differences in the three basal media and from the differences in concentration of the various compounds studied did not alter the thermocouple readings to an appreciable extent (Corrections were necessary for the liver extract which is colored) By vigorous mechanical shaking of the yeast suspensions after the 6 hour growth period in the apparatus it was shown that the cells were not clustered enough to significantly affect the opacity of the suspensions

The pH of 4.8 produced by the  $\text{KH}_2\text{PO}_4$  buffer was chosen because it is approximately the optimum for yeast (12), and a pH this low reduces preformed  $\text{CO}_2$  to a negligible amount (13) It was also found that in this region a small variation in pH does not affect the  $\text{CO}_2$  constant of the apparatus Closer to neutrality  $\text{CO}_2$  is much more soluble and a slight variation in pH is more serious

Table I shows the effects of the three most carefully studied materials on the respiration and growth of the deficient G I yeast.

The large number of preliminary experiments that were run made it possible to plan the final series of runs so that they could be completed in a short period thereby eliminating much of the variations resulting from changes in the stock yeast. The data listed within a horizontal column were obtained (except for vertical column F) in one run and fine comparisons among them are valid. In order to make comparisons of the data listed in vertical columns as accurate

TABLE I  
Deficient G M<sub>1</sub> Yeast

	A	B	C	D	E	F	G
CO <sub>2</sub> evolved—mm <sup>3</sup> per mg per hour at end of 1 hr							
Medium 1—sucrose*	23 4	25 8	39 6	22 6	23 7	53 5	129 0
Medium 2—sucrose + salts	35 7	43 0	53 0	38 4	33 2	67 0	150 0
Medium 3—sucrose + salts + asparagine	55 3	73 0	84 0	60 0	76 2	93 0	104 0
O <sub>2</sub> absorbed—mm <sup>3</sup> per mg per hour at end of 1 hr							
Medium 1—sucrose*	2 6	2 2	3 2	2 5	2 5	3 0	7 2
Medium 2—sucrose + salts	3 9	6 1	6 3	5 6	4 4	5 1	14 6
Medium 3—sucrose + salts + asparagine	5 5	5 3	8 4	5 3	6 3	5 5	11 0
Ratio of crop to seeding at end of 6 hrs							
Medium 1—sucrose*	1 0	1 0	1 1	1 0	1 0	3 1	3 5
Medium 2—sucrose + salts	1 0	1 0	1 1	1 0	1 05	3 8	5 8
Medium 3—sucrose + salts + asparagine	1 0	1 4	1 6	1 0	1 1	4 2	7 1

\* All three media were buffered with KH<sub>2</sub>PO<sub>4</sub>.

Vertical column A is with no added stimulant, B is with 0.1  $\gamma$  pantothenic acid, C is with 10  $\gamma$  pantothenic acid, D is with 0.1  $\gamma$  thiamin, E is with 0.1  $\gamma$  pantothenic acid and 0.1  $\gamma$  thiamin, F is with 10 mg liver extract No. 343, and G is with 100 mg liver extract No. 343.

as possible, a factor was used to eliminate the variation in the controls. Rates of CO<sub>2</sub> evolution and O<sub>2</sub> consumption 1 hour after the end of the equilibration period are given in cubic millimeters per hour per milligram of yeast. Curves plotted from readings over a period of the first 3 hours showed that rate at the end of 1 hour was a fair basis of comparison. Growth is given as ratio of crop to seeding at the end of 6 hours. Ten gammas of pantothenic acid (calculated from the preparation of known purity), 0.1 gamma thiamin, and 100

mg liver extract were found to be the optimal concentrations. In order to further correlate the effects certain other concentrations as well as combinations of stimulants were studied.

Columns B and C show the great stimulative power of pantothenic acid on  $\text{CO}_2$  evolution,  $\text{O}_2$  consumption, and growth. It is evident that 10 gammas of pantothenic acid increased the rate of  $\text{CO}_2$  evolution in medium 1 about 70 per cent at the end of 1 hour. After 4 hours the  $\text{CO}_2$  evolution was stimulated over 150 per cent although little growth occurred. A similar effect was shown in media 2 and 3, except that in the presence of the asparagine there was much more growth. It will be noted that in the absence of pantothenic acid (or the liver extract which contains it and other yeast nutritives) no growth was evidenced. It is apparent therefore that the respiration and growth stimulative functions of the acid can be separated by the proper choice of media. This is in line with the findings of others (14-16) that respiration and growth may be quite distinct. Still more evidence that this is so is found in column A, where it is shown that adding the inorganic salts to medium 1 or asparagine to medium 2 greatly increases the respiratory rate without appreciably affecting the growth rate.

Under the conditions used the effect of pantothenic acid was far in excess of that of any of the other nine compounds studied. The only compounds which approached its effect are thiamin, ethanalamine (which must be added in relatively high concentrations), and  $\beta$  alanine, which is intimately related to pantothenic acid (10).

Very little effect was shown by the pantothenic acid on the regular Fleischmann cake yeast. Apparently this yeast already contains approximately the optimal concentration of the acid. This interpretation was substantiated by a series of experiments, not reported in detail, in which it was found that when the Fleischmann yeast was grown in a medium deficient in "growth substances" its respiration was greatly stimulated by pantothenic acid.

The optimal concentration of thiamin has a definite accelerative action on the respiration of the deficient G M yeast. It did not stimulate growth, in fact it inhibited the growth stimulative effect of pantothenic acid in medium 3. It also inhibited the respiratory stimulative effect of pantothenic acid in medium 2. Thiamin gave



about the same stimulative effects on the regular Fleischmann yeast as it did on the deficient G M yeast. Thus its effects appear to be of contrasting nature to those of pantothenic acid as regards both growth and respiration.

In all three sections of the table the striking effect of the liver extract is obvious. It raised the respiration rate and the growth far beyond the optimum obtainable with pantothenic acid and thiamin. Its activity on the regular Fleischmann yeast was of similar nature and magnitude. Liver extract therefore contains important respiration and growth stimulants other than pantothenic acid or thiamin.

The general agreement of results on the two yeasts becomes more important when it is realized that they are very different in the character and rate of their respiratory activity. The  $\text{CO}_2$  evolution of the regular Fleischmann yeast was roughly three times and the  $\text{O}_2$  consumption was nearly ten times that of the deficient G M yeast.

Consequently the respiratory quotient  $\left(\frac{\text{CO}_2}{\text{O}_2}\right)$  of the Fleischmann was approximately one-third that of the G M yeast.

### III

#### *Effects of Fermentation by Enzyme Preparations from Yeast*

Since the effect of pantothenic acid on respiration is not dependent on its effect on growth it was thought desirable to determine if the acid had an effect on fermentation by non-living systems.

A maceration juice prepared according to a procedure adapted from von Lebedev (17) was used. 1 kg of fresh Fleischmann baker's yeast (pound cakes) was washed 12 hours by allowing tap water to run over it in a large evaporating dish. After filtering at the suction pump the yeast was broken into small pieces, spread out in a thin layer, and dried 48 hours at  $30^\circ$ . Twice during the drying the yeast was screened, first through a 2 mm, then through a 1 mm screen. An electric fan was used during the last 10 hours of drying. The product (180 gm) was kept in a desiccator. The maceration juice was prepared by adding 3 volumes  $\text{H}_2\text{O}$  to the dry product and warming at  $35^\circ$  for 2 hours. 3 more volumes  $\text{H}_2\text{O}$  were then added and the cells filtered off (using ordinary filter paper). 1 cc of the filtrate was pipetted directly, or after dialysis, into the Warburg flasks. Solutions of sucrose,  $\text{KH}_2\text{PO}_4$ , and the pantothenic acid were then added in such volumes and concentrations that the final volume of 2.4 cc was 0.1 M in sucrose and 0.1 M in  $\text{KH}_2\text{PO}_4$ .

Although extensive experimentation was done no definite effect of pantothenic acid on the undialyzed juice (except inhibition at high concentration) could be detected over a period of several hours. Since pantothenic acid is known to be a small molecule and readily dialyzable (1) it appeared that it might be possible to dialyze out the pantothenic acid known to be present in the maceration juice. Thus it might be possible to obtain an enzyme system deficient in pantothenic acid. Consequently the maceration juice was dialyzed against running tap water for various lengths of time. Both sausage

TABLE II  
Net Gas Exchange—CO<sub>2</sub> Evolved Minus O<sub>2</sub> Absorbed

	First 1/4 hr	Sec nd 1/4 hr	Third 1/4 hr
<b>Yeast maceration juice dialyzed 1/2 hr</b>			
Control	10	11	11
Control + 1/10 γ pantothenic acid	12	13	12
Control + 1 γ	14	12	12
Control + 10 γ	12	12	13
<b>Yeast maceration juice dialyzed 1 1/4 hr</b>			
Control	7	8	9
Control + 1/100 γ pantothenic acid	10	10	9
Control + 1/10 γ	12	11	10
Control + 1 γ	9	9	8

Figures represent pressure differences in millimeters Brodie solution

casing and cellophane membranes were used with results qualitatively the same

The results obtained on a typical run with juice dialyzed  $\frac{1}{2}$  and  $1\frac{1}{2}$  hours are shown in Table II. Results are given directly in terms of pressure changes (millimeters of Brodie solution) for the first three 15 minute periods after the end of the equilibration period. Figures represent net gas exchange ( $\text{CO}_2$  evolved minus  $\text{O}_2$  absorbed), since preliminary runs showed that the pantothenic acid stimulated chiefly by increasing the  $\text{CO}_2$  output making it unnecessary to determine  $\text{CO}_2$  and  $\text{O}_2$  in separate flasks. Although the data show that fermentation was quite slow, duplicates agreed well for about 1 hour.

and a 1 mm difference is significant. The manometer constants were so nearly equal as to have a negligible effect on the relative values for these small readings

It is evident from the table that pantothenic acid is a potent stimulant for the enzymatic fermentation in the dialyzed maceration juice. Particularly striking is the effect shown by only 1/100 gamma of pantothenic acid on the juice dialyzed  $1\frac{1}{2}$  hours. The action occurs immediately and lasts less than an hour. A much larger per cent stimulation is shown if the first reading is taken after only 5 minutes instead of 15. It also seems probable that the effect could be still further increased by adding the pantothenic acid from the sidearm at the end of the 10 minute equilibration period.

Fermentation is known to be a very complex process requiring many "factors," some of which are dialyzable. Consequently it seems probable that the enzyme system is deficient in other essential factors besides pantothenic acid. Evidence supporting this viewpoint was obtained by adding  $\text{MgSO}_4$ ,  $\text{MnSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and acetaldehyde in concentrations calculated from those used by Warburg in some of his work on similar systems (18). The effective concentration of these compounds was an entirely higher order of magnitude than the concentration of pantothenic acid. The addition of these compounds increased the rate of fermentation 10-20 per cent and the further increase in rate by adding pantothenic acid to this modified control was essentially the same as on the untreated control.

Corroboration of these results was obtained in numerous experiments on the effects of pantothenic acid on zymoin (the "acetone yeast" of Harden (19)). As was to be expected from the work of others (20-22) it was found that various organic and inorganic compounds stimulated the fermentation by zymoin. The large effects of these compounds, together with the fact that relatively large concentrations of the pantothenic acid were required to give the maximum rate of fermentation, made it difficult to establish the effect of pantothenic acid itself. However, it was proved that the free acid (as well as the calcium salt) markedly supplemented the effects of the other compounds and made it possible to obtain a higher maximum rate of fermentation than was obtainable without the addition of pantothenic acid.

## IV

*Effects on the Respiration of Potato and Apple Tissue*

Since the importance of pantothenic acid to the respiration of yeasts and enzyme preparations from yeast seemed well established, the next step was to determine if the acid had an influence on the respiration of higher plants

The procedure used in this part of the investigation was adapted from that used by Lemmon in his careful study of the effects of pH on the respiration of potato tissue (23) Nine cylinders of tissue (three from each of three potatoes or apples) approximately 4 by 10 mm were cut as rapidly as possible and placed in each Warburg

TABLE III

	First 1/4 hr	Second 1/4 hr	Third 1/4 hr
Oxygen consumption of potato tissue			
Control	16 0	17 0	16 5
Control + 1/10 $\gamma$ pantothenic acid	18 5	18 5	18 0
Control + 1 $\gamma$ " "	18 0	18 5	16 0
Oxygen consumption of apple tissue			
Control	5 5	10 5	10 0
Control + 1/10 $\gamma$ pantothenic acid	9 0	11 5	10 5
Control + 1 $\gamma$ " "	7 5	11 5	8 5

Figures represent pressure differences in millimeters Brodie solution

flask in 2 cc of 0.1 M phosphate buffer at pH of 5.3 The pantothenic acid was added in 0.4 cc H<sub>2</sub>O The potatoes and apples were kept at 30° overnight before use Duplicates were always run and the results averaged The duplicates usually agreed to within less than 5 per cent during the first hour

In Table III are given a typical set of results for apple tissue and a similar set for potato tissue The numerous preliminary experiments showed that pantothenic acid did not appreciably affect the respiratory quotient  $\left(\frac{\text{CO}_2}{\text{O}_2}\right)$  Therefore the work was expedited by determining the O<sub>2</sub> consumption only As in the previous section the figures

represent millimeters pressure change for the three 15 minute periods immediately following the end of the equilibration period. The larger per cent stimulation by the pantothenic acid on the apple tissue may be due to the fact that the work on potato tissue was done first and the acid was added before the flasks were put on the manometers, while in the work on apple tissue the pantothenic acid was added from the sidearm of the flasks at the end of the 10 minute equilibration period.

The table shows that, as in the case of the enzyme preparations, the pantothenic acid has a strong stimulative action which begins immediately and lasts less than an hour. It is, therefore, evident that pantothenic acid is a potent stimulant for the respiration of these tissues. It is noteworthy that these tissues were not made "deficient" as was done with the living yeast and also with the enzyme preparations.

Considerable work has been done to determine if pantothenic acid has a stimulative effect on the respiration of sliced rabbit muscle and homogenized (and dialyzed) rabbit brain. Positive results have been indicated, but there are large experimental variations. It may be that larger effects were not obtained because pantothenic acid is tied up in the tissues (24). If pantothenic acid is present in optimal amounts it would be difficult to obtain a deficient tissue on which its effect could be demonstrated. It is planned to continue the investigation in this direction.

#### SUMMARY

Experiments using the Warburg-Barcroft apparatus led to the following results and conclusions: (1) Two yeasts in three different media were strikingly stimulated in their respiration by minute amounts of pantothenic acid. (2) Nine other compounds (vitamins and other biologically important substances) were tested and found in all cases to have on the deficient G M yeast, lesser and in some cases no appreciable stimulative effect. Thiamin was the most effective of these compounds. Its action was shown to be different and in some ways antagonistic to that of pantothenic acid. (3) Liver extract (Lilly's Number 343) contains substances capable of speeding up respiration (and growth) to a much higher level than

seems possible with known compounds (4) Pantothenic acid was found to have a definite stimulative effect on fermentation by dialyzed maceration juice from yeast (5) It likewise stimulated respiration of apple and potato tissue and indications of a similar effect on certain animal tissues were obtained

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# ELECTRIC IMPEDANCE OF THE SQUID GIANT AXON DURING ACTIVITY\*

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The permeability of a membrane to a penetrating substance is given quantitatively by the amount of the substance which crosses a unit area of the membrane in unit time under the action of a unit force. In simple cases of ionized substances both the amount of substance and the force acting may be expressed in electrical terms. Then the permeability may be ultimately converted into coulombs per second for a square centimeter and a potential difference of 1 volt, which is the conductance, in reciprocal ohms, for a square centimeter. Marine eggs have been measured before and after fertilization and a number of tissues have been measured during activity, but the attempts to interpret the observed conductance changes have not been particularly satisfactory. Since it is quite generally believed that the depolarization of a nerve fiber membrane, during excitation and propagation, involves an increased permeability to ions there have been many attempts to detect and to measure this change as an increase in the electrical conductivity. A decrease in the longitudinal low frequency impedance of frog sciatic nerve during activity was found by Lullies (1930) (also Cole and Curtis, 1936), and a similar change was found in the transverse impedance of the squid giant fiber (Curtis and Cole, 1938). In these cases the measuring current was also the stimulating current and it was not possible to analyze the changes satisfactorily. In *Nitella*, Blinks (1936) showed with direct current transients that on excitation the membrane impedance decreased under the cathode, but it was not possible to separate the change into resistance and capacity components.

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Recently the transverse alternating current impedance of *Nitella* has been measured during the passage of an impulse which originated several centimeters away (Cole and Curtis, 1938 *b*). These measurements showed that the membrane capacity decreased 15 per cent or less while the membrane conductance increased to about 200 times its resting value. Also this conductance increase and the membrane electromotive force decrease occurred at nearly the same time, which was late in the rising phase of the monophasic action potential. Similar measurements have now been made on Young's giant nerve fiber preparation from the squid (Young, 1936). These were undertaken first, to determine whether or not a functional nerve propagates an impulse in a manner similar to *Nitella*, and second, because the microscopic structure of the squid axon corresponds considerably better than that of *Nitella* to the postulates upon which the measurements are interpreted.

The squid axon has about the same membrane capacity (Curtis and Cole, 1938) as other nerve fibers (Cole and Curtis, 1936) and *Nitella* (Curtis and Cole, 1937). The large diameter of the axon, 0.5 mm or more, makes it particularly favorable material since, for a given membrane conductance change, the magnitude of the observed transverse impedance change is proportional to the fiber diameter. It is also relatively easy to obtain considerable lengths of the axon which can be kept functional for hours.

The experimental procedure and the technique of analysis are fundamentally the same as those used for *Nitella* during activity, although variations have been necessary or possible because of the relatively short time intervals involved. We will present and discuss here only observations made during the passage of an impulse which has been initiated at a distant point.

#### *Material and Dissection*

The Atlantic squid, *Loligo pealii*, was used at Woods Hole for these experiments. From early May until late June excellent animals were available, but later they were smaller, not so numerous, and did not live long in the aquarium. The measuring cell was designed for an axon diameter between  $530\mu$  and  $580\mu$  and this was usually to be found in squid having a mantle length between  $10\frac{1}{2}$  and  $11\frac{1}{2}$  inches. Slender animals were preferred because the axons were of nearly uniform diameter over their usable length.

It seemed fairly certain that body fluids had an injurious effect, so the mantle was removed from the rest of the animal under running sea water. It was slit along the ventral mid line, and laid out flat. The preganglionic stellate and fin nerves were then cut on each side and the mantle freed from the rest of the body. The mantle was placed on a large glass absorption cell cooled by circulating water and illuminated from underneath, and the hindmost stellar nerve dissected out. The nerve was freed at the stellate ganglion, ligated with silk thread, and then separated from the fin nerve and the mantle up to the point where it entered the muscle. It was again ligated and cut free. The small fibers were then teased away from the giant axon in sea water in a Petri dish. The two silk threads were held against the bottom by clips on opposite sides of the dish after the nerve had been stretched until the giant axon was nearly straight. Under a binocular dissecting microscope, the small fibers were all cut near one end with a sharp pointed double edged scalpel and then pulled slightly to one side and cut free where they looped around the giant axon. The axon has a number of small branches which must be cut at a short distance from it. These can often be pulled free without immediately killing the nerve, but degeneration will usually progress slowly from that point. The fibers which remained in good condition for considerable time were usually very turgid after dissection. A 3 cm length of axon was necessary but it was usually possible to get 6 or 8 cm in good condition.

After the axon was placed in the measuring cell the sea water circulation was started, and preliminary measurements made. If the impedance, the threshold for excitation and the impedance change on excitation became constant within an hour measurements were started. If not the fiber was discarded because experience showed that the impedance and impedance change would decrease and the threshold for excitation would increase more or less steadily until after 4 to 6 hours the fiber failed. Under favorable conditions, the axons would have quite constant electrical characteristics for 6 to 8 hours and one remained excitable for 36 hours.

Some experiments were made at room temperature but the majority were between 2°C and 4°C where the conduction velocity was less, a higher bridge input and consequent greater sensitivity could be used, and the axon survival was better. When monophasic action potentials were desired, one end of the axon was dipped in iso-osmotic KCl for a few minutes.

### *Measuring Cell*

The measuring cell shown in Fig. 1, is very similar to that used for *Nitella*. The axon was placed in the trough 570 $\mu$  wide and 560 $\mu$  deep, cut in the top of a sheet of Victron. The entire cell was mounted on a metal box which was maintained at constant temperature by circulating water. After the axon was in place, the cell was covered with a thin microscope cover glass and sea water circulation was started through the cell by a siphon. This maintained a slight negative pressure in the trough and so held the cover glass in place. The cell assembly was mounted on the bridge panel in an insulating shield to lessen temperature changes.

The stimulating electrodes,  $a$ , impedance electrodes,  $b$ ,  $b'$ , and potential electrodes,  $c$ ,  $c'$ , were all platinized platinum. The impedance electrodes should be wide to minimize electrode polarization corrections at low frequencies and the effect of the "fringing" of the current at the electrode edges, but narrow to include as short a length of the axon as possible. The electrode width of  $570\mu$  was a fairly satisfactory compromise. The polarization was considerable below 1 kc,<sup>1</sup> but the duration of the impedance change was short enough to make the interpretation of lower frequency measurements difficult, and the time of transit of a given point of the impulse past the electrode region was about 0.1 millisecond which was too short for the bridge amplifier to follow faithfully. The electrode wells used for the resting squid axon (Curtis and Cole, 1938) were not tried because of the loss in sensitivity and difficulty of construction involved.

The potential electrodes,  $c$ ,  $c'$ , were all  $140\mu$  wide. The "monophasic" potentials,  $V$ , were measured between the grounded impedance electrode,  $b$ , and the farther  $c'$  electrode on an inactive portion of the axon (see Fig 9 a).

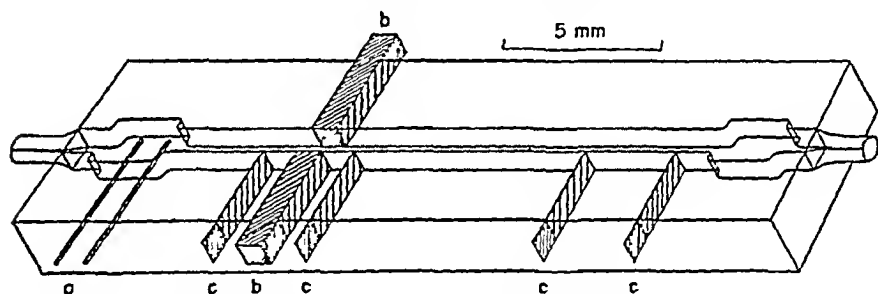


FIG 1 Measuring cell for squid giant axon. The central trough is for the axon and the connections for circulating sea water are at each end. The axon is stimulated with electrodes,  $a$ , the transverse impedance measured between electrodes,  $b$ ,  $b'$ , and the action potentials between various combinations of  $b$ ,  $c$ , and  $c'$ .

On the basis of simple cable theory which has been discussed (*Nitella*<sup>2</sup>), the monophasic action potential is proportional to the potential difference across the membrane and its slope or first derivative is proportional to the current flow parallel to the fiber axis. The density of current flow across the membrane is then given by the second derivative. For *Nitella*, the first and second derivatives were usually calculated from the monophasic action potential, but this was not a particularly satisfactory procedure. The disadvantages were even greater with the squid axon. The oscillograph time scale was not linear and completely monophasic action potentials were seldom obtained, so the extra potential electrodes were added to record the approximate derivatives directly. As is shown on p 663, the potential difference between electrodes  $c$ ,  $c'$  (see Fig 9 b) is approxi-

<sup>1</sup> Kilocycles per second

<sup>2</sup> Cole, K. S., and Curtis, H. J., 1938-39, *J Gen Physiol*, 22, 58

mately the first derivative,<sup>3</sup>  $V_i$ , of the potential at the impedance electrodes. Also the potential difference between the mid point of a high resistance across the electrodes  $c, c$  and the ground electrode  $b$  (see Fig. 9c) gives the approximate second derivative,<sup>3</sup>  $V_{ii}$  at the same point.

Conduction velocities were measured by the separation between the  $V_i$  potential at electrodes  $c, c$  and that at  $c', c'$  when these were recorded on the same film. If there was an inactive end under one  $c'$  electrode, the  $V_i$  potentials between this electrode and each of the  $c$  electrodes, were used to determine the conduction velocity.

### Electrical Apparatus

The ellipse and motion picture technique, as used for the *Nitella* experiments, gave complete information on each excitation and would have been ideal for these experiments if the short duration of the action had not made it completely impractical. The next best system considered was the "Lichtbandmethode" of Hőzawa (1935), using the Schering bridge in which either the reactance or resistance component of an impedance change can be recorded independently of the other component. The objections to its use were primarily practical ones for, after it was recognized that the Wheatstone bridge could be used, it was obviously inadvisable to attempt to design, construct, and learn to operate a new bridge for the investigation of a phenomenon that was not known to exist. The alternating current Wheatstone bridge which was used and the method of making steady state impedance measurements with it have been described (Cole and Curtis, 1937). A schematic diagram of the equipment is shown in Fig. 2.

The strength of the bridge current was kept as low as possible because it was found that even relatively small currents can cause local changes in the portion of the axon between the impedance electrodes. By maintaining 50 to 100 mv across the impedance electrodes for a number of minutes, the impedance change on excitation was diminished and only a partial recovery was possible. But if the axon was then moved along the trough so that a different section was between the electrodes the impedance change was as large as ever. During a run the voltage across the axon was not allowed to exceed 20 mv and this was maintained for as short a time as possible.

As a result of the low input voltage requirement, an adequate sensitivity could be obtained only by the use of considerable detector amplification. An ordinary audio frequency bridge output amplifier was satisfactory for *Nitella* which has a transverse characteristic frequency<sup>4</sup> of 1 kc but this did not go to high enough frequencies for the squid axon where the characteristic frequency is about 30 kc. The amplifier was first replaced by a conventional radio superheterodyne mixer, to convert the bridge output to 175 kc, and a transformer coupled amplifier tuned to this frequency. This arrangement introduced very serious distortion when the

<sup>3</sup>  $V_i = \partial V / \partial t$      $V_{ii} = \partial^2 V / \partial x^2$

<sup>4</sup> The frequency at which the series reactance is a maximum (Cole 1932)

amplifier was sharply tuned so that a bridge frequency as low as 20 kc could be used. A balanced modulator was then substituted for the simple mixer and the amplifier tuning broadened as much as possible. A bridge frequency of 2 kc could then be used and the distortion was greatly decreased.

A differential resistance-capacity coupled amplifier with degeneration in the common mode was used for the action potentials. The output of either this amplifier or the 175 kc bridge amplifier could be switched to the vertical deflecting plates of the cathode ray oscillograph through a single stage untuned power amplifier.

The conversion of all bridge output frequencies to 175 kc before they were impressed on the oscillograph as well as the short time intervals involved precluded

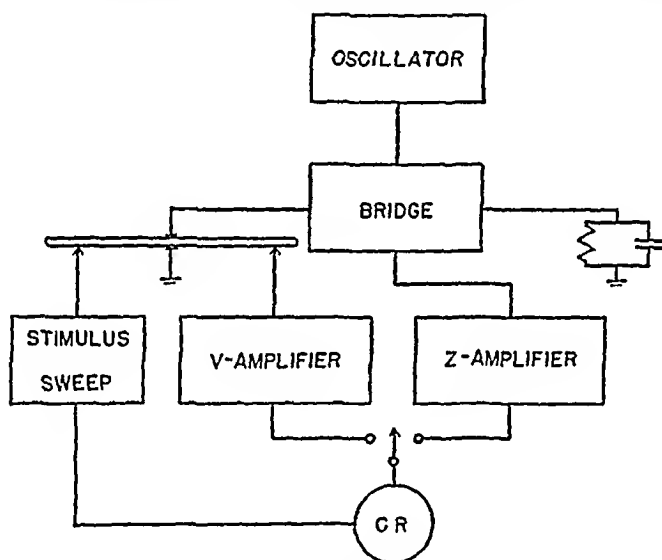


FIG 2 Schematic diagram of the electrical equipment. The axon is at the left, and the balancing resistance and capacity at the right, of the bridge. The action potential and bridge amplifiers are represented by V-amplifier and Z-amplifier respectively and the cathode ray oscillograph by C R.

the use of the *Nitella* motion picture and ellipse technique, but the use of a horizontal sweep circuit was convenient since the axon could be stimulated between one and ten times per second as was usually done. The stimulus was a short shock which was taken from the sweep circuit in such a manner that it was applied at the start of the sweep, and a shielded transformer was used in the stimulus circuit to reduce the shock artifact.

### Procedure

*Experimental* — After the axon was placed in the measuring cell and had become steady, the resting parallel resistance and capacity were measured at 9 frequencies

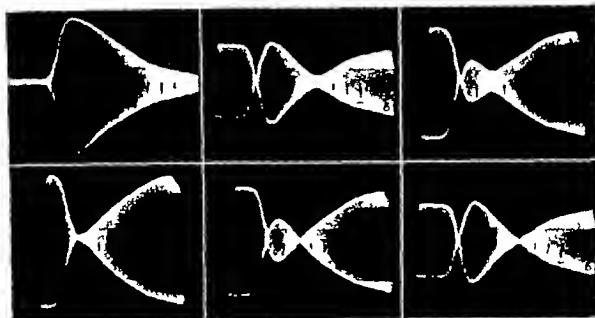


FIG 3 Bridge output during the passage of an impulse with the bridge balanced for the impedance of the axon first at rest and then at various times during the action. Frequency 50 kc. maximum change, 7 per cent.

from 2 kc to 1000 kc. At one frequency the bridge would then be balanced with the oscillograph which gave a narrow horizontal trace each sweep when the stimulus was below threshold. After the threshold was reached the bridge went off balance when the action came between the impedance electrode and the oscillograph line broadened into a band. Then as the axon recovered the bridge returned to balance and the band narrowed down to the resting line again as shown in Fig 4. The width of the band at any point is proportional to the magnitude of the change of impedance but does not give any information as to the relative values of the resistance and capacity components of this change. The resistance and capacity of the known arm of the bridge were then altered so that although the bridge is no longer balanced at rest it would be balanced at some particular point during the activity as shown in Fig 3. In this way the resistance and capacity were measured during the action at a series of ten or fifteen points along a scale on the face of the oscillograph which was calibrated in milliseconds.

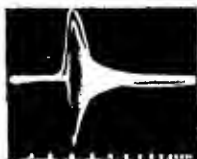


FIG 4 Double exposure of the 2 per cent maximum bridge unbalance at 20 kc and the monophasic action potential at one of the impedance electrodes. The time marks at the bottom are 1 millisecond apart.

In this way the resistance and capacity were measured during the action at a series of ten or fifteen points along a scale on the face of the oscillograph which was calibrated in milliseconds.

The relation between the impedance change and the action potential was recorded photographically as shown in Fig 4 (Cole and Curtis 1938 a). The impedance change was first exposed for about ten sweeps the oscillograph was then

switched from the bridge amplifier to action potential amplifier, the bridge oscillator turned off, and without change of the sweep circuit or stimulus, a second exposure of two or three sweeps of the action potential was made a second later on the same film. The resistance and capacity were also measured both at rest and at the time of the maximum impedance change.

These measurements during activity were made at frequencies of 5, 10, 20, 50, 100 kc and sometimes 200 and 500 kc. Finally, another complete frequency run was taken on the axon at rest to show what changes had taken place during the experiment, and if these were too large the experiment was discarded. The conduction velocity and the amplification of the action potential amplifier were measured and a time record was made to calibrate the sweep circuit.

When the axon was removed from the cell at the end of the experiment it was carefully examined and the diameter measured at the point which was between the impedance electrodes. The cell was filled with sea water and resistance and capacity data were taken at low frequencies, to determine the electrode polarization, and at high frequencies, to measure the static capacity of the cell.

*Analytical*—The data on parallel resistance and capacity,  $R_p$  and  $C_p$ , were corrected for the electrode polarization and static capacity of the cell (Cole and Curtis, 1937) and the series resistance and reactance,  $R_s$  and  $X_s$ , were calculated by

$$R_s = R_p / (1 + R_p^2 C_p^2 \omega^2) \quad X_s = R_p^2 C_p \omega / (1 + R_p^2 C_p^2 \omega^2)$$

The frequency impedance locus, which is the path followed when  $X_s$  is plotted against  $R_s$ , as the frequency of the measuring current is varied (Cole, 1928, 1932), was plotted for the resting fiber (Fig. 5). The properties of the resting axon have been calculated from the extensions of the Rayleigh equation which have been used for single cylindrical cells (Curtis and Cole, 1937, 1938, Cole and Curtis, 1938 b). The average membrane capacity at 1 kc is  $1.80 \mu\text{f}/\text{cm}^2$  with an average phase angle of  $71^\circ$ . The average internal specific resistance is 71 ohms cm or about 2.9 times that of sea water.

At each frequency, the value of  $R_s$  and  $X_s$  during activity may be plotted in the same manner and trace a path which is called the time impedance locus. The points of maximum change at five frequencies are shown in Fig. 5 and all of the 10 kc points taken at different times during the action of another axon are shown on a larger scale in Fig. 6. If the only change during activity were a decrease of membrane resistance, then the time impedance locus at each frequency would be

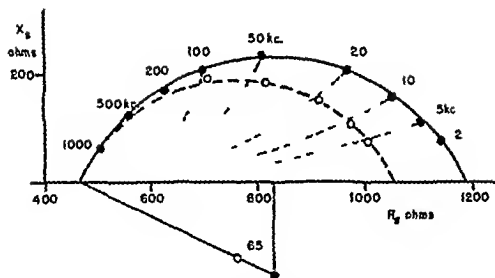


FIG 5 Impedance loci of series resistance,  $R$ , vs series reactance,  $X_s$  in ohms. The solid circles which lie on the solid frequency locus are obtained from resting nerve at the indicated frequencies, and the open circles are the points of maximum change which lie on the heavy broken line representing the frequency locus. The light broken lines forming circular arcs are the theoretical time loci for a pure membrane resistance decrease at each of five frequencies.

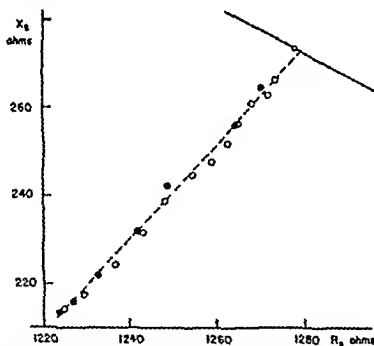


FIG 6 Time impedance locus of series resistance,  $R$ , vs series reactance,  $X_s$ , at 10 kc during the passage of an impulse. The solid circles were taken during the impedance decrease on excitation and the open circles during the increase on recovery. The broken line represents part of a circular arc which is the theoretical locus for a pure membrane resistance change and the solid line is a portion of the resting frequency locus.



an arc of a circle passing through the resting point and tangent to the resistance axis at the infinite frequency extrapolation as shown in equation (8) and Fig 11 of the *Nitella* paper<sup>5</sup> This is true as a rough approximation and the minimum membrane resistances have been calculated by *Nitella* equation (6) These minimum values range from 14.7 to 53.5 ohm cm<sup>2</sup> with an average of 28 ohm cm<sup>2</sup> This minimum value is independent of frequency and it is further found that the time variation of the membrane resistance during the passage of the impulse is approximately the same for all frequencies

The departures from these arcs are then due to a decrease of the membrane capacity during activity, which can be calculated by *Nitella* equation (9) The capacity decrease in Fig 5 depends upon the frequency and reaches a maximum of nearly 10 per cent at 50 kc At the other extreme is the negligible capacity change which was found from the data in Fig 6 The average capacity decrease for all experiments is about 2 per cent It is usually found, as can be seen in Fig 3 that the bridge balance is not the same on the rising and falling portions of the impedance change and this means that the time locus does not retrace its initial path during recovery Because of this the calculated capacity decrease lags slightly behind the resistance decrease during the passage of the impulse On the other hand the impedance change records made at different frequencies are practically identical This means that the apparent capacity change either depends upon frequency or is an artifact introduced by the bridge and its amplifier The latter possibility will be considered later (p 661)

It is convenient to work with the oscillograph records of the impedance change as far as possible Since bridge balance measure-

<sup>5</sup> In the *Nitella* paper, the sentence which includes equation (8) is incorrect and should read as follows, "By eliminating  $r_1$  we find

$$r^2 + x^2 - 2r_\infty r - [(\bar{r} - r_\infty)^2 + \bar{x}^2]x/\bar{x} + r_\infty^2 = 0$$

which is the equation of a circle having its center at the point,

$$r_\infty, [(\bar{r} - r_\infty)^2 + \bar{x}^2]/2\bar{x}$$

and the radius,

$$[(\bar{r} - r_\infty)^2 + \bar{x}^2]/2\bar{x}''$$

ments during the impulse indicate that the change of membrane capacity is small, equation (4) shows that the width of the impedance band on the oscillograph should be approximately proportional to the change of membrane conductance. It has been found experimentally that both the "balance" and "unbalance" data give nearly the same time course for the membrane conductance. The average time of rise to maximum conductance is between 250 and 300  $\mu\text{sec}$ , but this value cannot be accepted until its accuracy has been established (see below).

### *Distortions and Corrections*

The ideal measuring equipment would record accurately the properties of an infinitesimal length of axon, regardless of adjacent portions and independent of what happened the instant before. But in these experiments the impedance and the action potential have been measured over at least a half millimeter length of axon, while the responses of the bridge and the amplifiers may be expected to lag behind the phenomena.

Lines of current flow resulting from the action potential which would otherwise be confined to the sea water in the trough will enter and leave the impedance electrodes as the action passes them. This may alter the speed of propagation (Hodgkin, 1939) and modify other characteristics of the impulse in this region but no attempt has been made to estimate the magnitude of these effects.

*Impedance*—The effects of electrode length and current spread on the impedance measurements are the same as for the *Nitella* cell. If a perfectly sharp change of membrane conductance moved between the impedance electrodes, the observed change of impedance would be spread over the entire effective electrode length, including the region of current spread beyond the ends of the electrodes. As for the *Nitella* cell, this effect has been investigated by measuring the resistance as a cylindrical glass rod was moved along the trough with its end passing through the electrode region. The square cut end of the rod was then equivalent to the sharp transition from a non conducting to a conducting fiber which corresponded to a sudden drop of membrane resistance.

The results of Fig. 7 show that although the actual electrode length was 570  $\mu$  and 90 per cent of the change was confined to 1000  $\mu$ , the entire electrode region was over 1600  $\mu$  long. If the rod moved at 12 meters per second, the 90 per cent change would require 83  $\mu\text{sec}$  and the whole over 130  $\mu\text{sec}$ . This is a considerable portion of the time of rise of the impedance change as observed on axons having this velocity and so must be considered.

For a steady state, the deflection of the oscillograph was proportional to the difference between the impedances of the known and unknown arms of the bridge but the response to a rapid change of impedance must also be determined. After the bridge has been thrown off balance, the currents in the bridge oscillator, and

detector circuits will change only as fast as the inductances, capacities, and resistances will permit, and the voltage appearing across the detector will be distorted. Rough calculations indicate that under ideal conditions this effect would have a time constant of no more than a few micro-seconds. And when bridge output voltage is applied to the modulator and amplifier further distortion may be expected for, so far as changes of amplitude are concerned, this system is equivalent to an audio frequency amplifier. The combined characteristics of the bridge, modulator, and amplifier have been determined by recording the response to a sudden unbalance of the bridge. The bridge was balanced with a high resist-

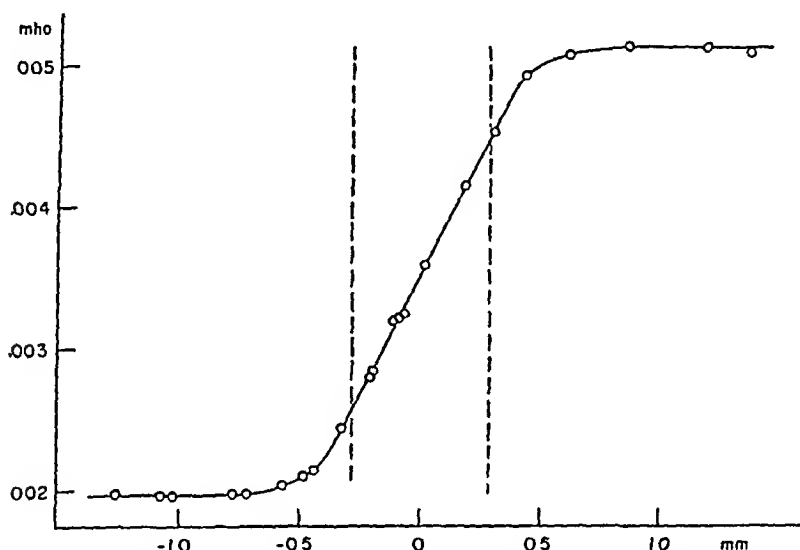


FIG 7 Conductance of the measuring cell, with a glass rod in place of the squid axon. The rod is introduced between the electrodes and is moved to the right so its left end goes through the electrode region. The electrode boundaries are given by the broken lines and the abscissae are distances in millimeters between the left end of the rod and the center of the electrodes.

ance shunt on the known arm of the bridge. One circuit on a Lucas spring rheotome then started the oscillograph sweep and a millisecond later a second circuit removed the shunt and so threw the bridge off balance. The record shown in Fig 8 a has a complicated form, which has not been satisfactorily explained, but it may be roughly approximated by an exponential having a time constant of 100  $\mu$  sec.

It would be difficult to obtain directly the oscillograph response to a sudden change of membrane conductance moving past the impedance electrodes with a velocity of 12 meters per second, but it has been calculated by combining the data

of Figs 7 and 8 *a* in Duhamel's integral or the principle of superposition<sup>6,7</sup> With the response of the oscillograph to a known cause, it is theoretically possible to determine the cause of any other effect but the practical difficulties are considerable and several methods for the correction of the experimental curves have been tried and found unsatisfactory. For example the desired curve may be approximated by an exponential with a time constant of 0.125 millisecond and the data corrected by the subtangent method (*cf.* Lucas 1912 Rushton 1937) as has been done in Fig 10. In general these corrections have shortened the apparent time of rise of the membrane conductance to 100  $\mu$ sec or less but have usually increased its maximum value by only about 10 per cent.

It should also be pointed out that the data obtained by balancing the bridge at different points of the impedance change are subject to the same distortions by the bridge modulator and amplifier. It is to be expected that there will be a phase alteration introduced during and for a short time after a rapid impedance change and this would require false capacity and resistance changes for the apparent balance point. The magnitude of this effect has not been investigated in detail and it is difficult to estimate how much of the apparent change of membrane capacity may be due to it.

**Action Potentials**—A strictly monophasic action potential was never obtained probably due to some remaining activity of the distant end or a lack of uniformity of the axon in the region between the two electrodes. For this reason and because it is preferable to make as many of the measurements in the immediate neighborhood of the impedance electrodes as possible the integral of the first derivative curve is more satisfactory. The first derivative curve is an approximation as is discussed on p 664. The two portions of the rising phase of the monophasic action potential have an average time constant of about 110  $\mu$ sec corresponding to a length of about 1.3 mm. This is very close to 1.25 mm the half separation of the  $V_i$  electrodes. Thus we find by equation (9) that the recorded  $V_i$  curve should be more than 60 per cent wider at the half maximum than the actual  $V_i$ . If we now differentiate this curve to obtain  $V_{ii}$  we compute by equation (10) that the two maxima would be separated by 2.5 mm or 210  $\mu$ sec if the rising portion is a double exponential whereas without distortion the reversal for this curve



FIG 8 (*a*) Response of the bridge and the bridge amplifier to a sudden unbalance at 20 kc (*b*) Response of the action potential amplifier to a suddenly applied potential

<sup>6</sup> Webster A. G. 1927 Partial differential equations of mathematical physics. Leipzig: Teubner. p. 172.

<sup>7</sup> Busb V. 1929 Operational circuit analysis. New York: John Wiley and Sons. p. 68.

should take place instantaneously. On the other hand, using the three electrode connection, the reversal time would be  $175 \mu\text{sec}$  and values of from 180 to  $200 \mu\text{sec}$  have been found. A simple method has not been found for applying corrections for the electrode separations, and no attempt has been made to construct any complete curves.

After the potential is on the electrodes, there is still the action potential amplifier distortion to consider. This also was determined with the spring rheotome which applied a known potential to input of the amplifier. The response curve shown in Fig. 8 *b*, is approximately an exponential with a time constant of  $80 \mu\text{sec}$ . The monophasic action potential record of Fig. 10 has been corrected for this time constant by the subtangent method.

### Theory

*Membrane Conductance*—The output voltage of the bridge and the oscillograph deflection are proportional to

$$Y = 2 \left| \frac{z - \bar{z}}{z + \bar{z}} \right|, \quad (1)$$

where  $\bar{z}$  is the impedance at balance and  $z$  is the impedance off balance. When the resting impedance is  $\bar{z}$  and the membrane conductance changes by an amount  $\lambda$  without change of membrane capacity, the impedance becomes by *Niella* equation (7),

$$z = \frac{a\sigma\lambda r_\infty + \bar{z}}{a\sigma\lambda + 1}, \quad (2)$$

where  $a$  is the fiber radius,  $\sigma$  is a complex constant involving  $\bar{z}$ , and  $r_\infty$  is the infinite frequency resistance. We then have, approximately,

$$Y = \frac{2\beta\lambda}{1 + \beta\lambda}, \quad (3)$$

where

$$\beta = \left| \frac{a\sigma(\bar{z} - r_\infty)}{2\bar{z}} \right|,$$

so if  $\beta\lambda$  is small the oscillograph deflection is proportional to the change of membrane conductance. In practice, the maximum change of conductance  $\lambda_0$  was determined from bridge balance measurements at rest and maximum change. If the oscillograph deflection at the maximum is  $Y_0$  then

$$Y_0 = \frac{2\beta\lambda_0}{1 + \beta\lambda_0},$$

and when  $\lambda$  is calculated by

$$\lambda = I \lambda_0 / I_0$$

the fractional error in this procedure is given by

$$\frac{I \lambda_0 / I_0 - \lambda}{\lambda} = \frac{I_0 - I}{2 - I_0} \quad (4)$$

The average value of  $I_0$  was about 8 per cent at low frequencies, so that the maximum error in the membrane conductance change was about 4 per cent for the smallest changes and became less nearer the maximum.

*Action Potential Derivatives*—At a particular instant let the action potential at each point,  $x$  along the axon be  $V(x)$ . The potential at a neighboring point,  $x + \delta$  is then given by Taylor's expansion,

$$V(x + \delta) = V(x) + \delta V'(x) + \frac{\delta^2}{2!} V''(x) + \frac{\delta^3}{3!} V'''(x) + \dots$$

where  $V$ ,  $V'$ , etc. are the first, second, etc. total derivatives with respect to the independent variable,  $x$  in this case. The difference in potential  $V_1$  between two electrodes at a distance on either side of the point  $x$  (see Fig. 9b) is

$$V_1 = V(x + \delta) - V(x - \delta) = 2\delta V'(x) + \frac{2\delta^3}{3!} V'''(x) + \dots \quad (5)$$

and if  $\delta$ ,  $V'$ , and successive derivatives are sufficiently small,

$$V_1 = 2\delta V'(x) \quad (6)$$

Thus the extreme case of a diphasic potential with electrodes close together is proportional to the first derivative,  $V'$ , with respect to moving coordinates and also with respect to time at a fixed point,  $V_t$ , if the velocity is constant.

Similarly when the circuit of Fig. 9c is used, the potential at the midpoint of the resistor connected between points  $x + \delta$ , and  $x - \delta$  is

$$\frac{1}{2}[V(x + \delta) + V(x - \delta)]$$

and the potential difference between this point and the center point  $x$  is

$$V_2 = \frac{1}{2}[V(x + \delta) + V(x - \delta)] - V(x) = -\frac{\delta^2}{2!} V''(x) + \frac{\delta^4}{4!} V^{(4)}(x) + \dots \quad (7)$$

which is proportional to  $V''(x)$  if  $\delta \ll \lambda$ , and successive derivatives are small or

$$V'' = \frac{\delta^2}{2!} V''''(x) \quad (8)$$

The question of how small  $\delta$  must be depends of course upon  $V(x)$  and we shall consider the errors which may be introduced in the rising phase. For purposes of calculation this part of  $V(x)$  will be assumed to be made up of two identical exponentials symmetrically placed

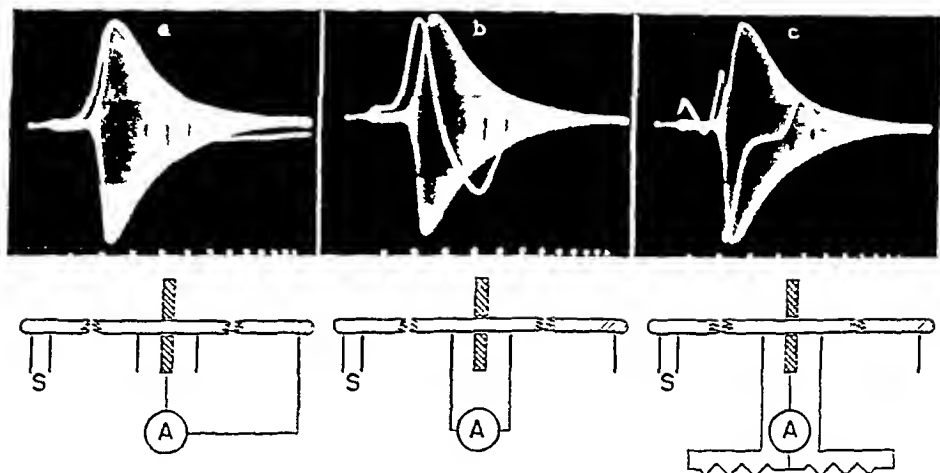


FIG. 9. Double exposures of the impedance change at 10 kc. on each with the action potential in each picture from the circuit shown below it. (a) is  $V$ , the monophasic potential, (b) is  $V'$ , the first derivative or axial current, and (c) is  $V''$ , the second derivative or membrane current. The time marks at the bottom are 1 millisecond apart.

with respect to the half maximum point which will be taken as the origin. Then on the upper half

$$V(x) = 1 - e^{-x/\lambda},$$

and on the lower

$$V(-x) = -(1 - e^{x/\lambda}),$$

where  $\lambda$  is the characteristic length. When  $x > \delta$

$$V(x + \delta) - V(x - \delta) = e^{-x/\lambda}(e^{\delta/\lambda} - e^{-\delta/\lambda}), \quad (9)$$

and if  $x$  is the point of half maximum of this potential

$$e^{x/\lambda} = e^{\delta/\lambda} + 1$$

If  $\delta/\lambda$  is small the width of the true  $\dot{V}$  at half maximum is increased by  $\delta$ , and when  $\delta = \lambda$  the width is 62 per cent too large. Obtaining  $\dot{V}$  by direct differentiation of this approximate  $\dot{V}$  we find that the maximum occurs at  $x = -\delta$  and the minimum at  $x = \delta$  so that the peaks are separated by  $2\delta$  whereas there should be a discontinuity with reversal of sign at  $x = 0$ . A similar error is found in the value of  $\dot{V}$  measured directly from the three electrodes, the minimum being at the value of  $x < \delta$  given by

$$\delta/\lambda = x/\lambda + \log \cosh x/\lambda \quad (10)$$

When  $\delta/\lambda$  is small the separation of the peaks is  $2\delta$  but when  $\delta = \lambda$  the separation is  $1.49\delta$ .

#### DISCUSSION AND CONCLUSIONS

The values for the capacity and phase angle of the membrane and the internal resistance of the resting axon given above are somewhat different from those which were found before (Curtis and Cole, 1938), but this is not particularly surprising. Although the material in the present work was in considerably better condition, the electrodes were short and the data were calculated on the assumption that end effects were negligible, and it was found for muscle that the measured phase angle was less for short electrodes than for long (Cole and Curtis, 1936). It has been assumed in calculations on transverse impedance measurements that the resting axon membrane is non-conducting. The value of  $1000 \text{ ohm cm}^2$  obtained by Cole and Hodgkin (1939), more than justifies that assumption. There are considerable differences between the values of the internal specific resistances which have been measured but we are not in a position to explain them.

Turning now to the impedance change during activity, it is first necessary to show that this is a real effect and not an artifact resulting from the stimulus or the action potential. The stimulus may be eliminated because the effect is entirely all-or-none. The bridge balance remains unchanged until the stimulus reaches threshold, and then the unbalance picture remains unaltered as the stimulus is further increased except that it moves forward with the action potential because the point of stimulation moves closer to the impedance elec



trodes Any possible effect due directly to the action potential is eliminated for several reasons The only action potential which would be effective is that appearing across the impedance electrodes, which should be zero in a perfect axon In practice this potential was always present, although it was small and did not come through the bridge amplifier However, it was never the same in any two axons and yet the impedance change always had the same form Most conclusive, however, is the fact that the amplitude of the unbalance picture was directly proportional to the bridge input voltage from the oscillator while the action potential was unaffected

In spite of the shortcomings of the apparatus and the difficulty of correcting for them, the general nature and magnitude of the impedance change seems quite certain The decrease of the extrapolated zero frequency resistance (Fig 5) might be due to a change of either the volume of the axon or the resistance of the external medium, but these factors should alter the extrapolated infinite frequency resistance which is unchanged The phase angle of the membrane is unchanged and the membrane capacity does not change alone because this would merely move each point along the resting curve Consequently we must assume that there is a change of the membrane resistance which falls from a resting value of  $1000 \text{ ohm cm}^2$  to a minimum value which is probably 10 per cent and perhaps 20 per cent below the average uncorrected value of  $28 \text{ ohm cm}^2$  The 2 per cent decrease of membrane capacity is of quite a different order of magnitude, but even this value should not be taken too seriously because there are indications that the actual change may be somewhat less

We may reason, as we did for *Nutella*, that the conductance is a measure of the ion permeable aspect of the membrane and we see that the maximum conductance is far from a complete permeability And indeed the capacity, which represents the ion impermeable portion of the membrane, has not been encroached upon by more than 2 per cent Thus if the change on excitation is uniform throughout the structure of the membrane it must be so delicate as to leave the capacity and phase angle nearly unchanged and conversely if there are drastic changes they must be confined to a small fraction of the membrane area The time constant of the resting membrane is of

the order of 1 millisecond which is equivalent (*Nitella*)<sup>8</sup> to a thousand ions per second crossing the membrane for each ion pair separated by the membrane, and the time constant of 0.03 millisecond at the maximum then gives a permeability of some thirty thousand ions per ion pair

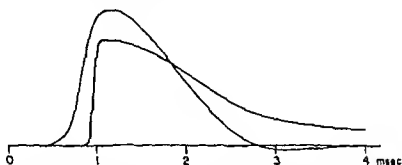


FIG 10 Membrane conductance increase (heavy line) after approximate corrections for electrode length and bridge amplifier response and monophasic action potential (light line) obtained from the first derivative after approximate correction for action potential amplifier response

The time course of the membrane conductance is best discussed in connection with the action potential, but before doing so we should submit some proof that the oscillograph sweep circuit which gave the horizontal time scale, did not alter in the interval between the impedance and action potential exposures. To do this, the two have been taken simultaneously by applying the  $V_i$  potential, of Fig 9 b, to the vertical plates and bridge output to the horizontal plates with the result shown in Fig 11. It is here seen as in Fig 9 b, that the potential rises to its maximum value before the bridge goes off balance. It is then clear that the conductance does not start to increase until the point of inflection on the  $V$  or membrane potential curve, Fig 9 a, which is the reversal point of the  $V_{ii}$  or membrane current curve, Fig 9 c. The small "foot" at the start of the membrane conductance pictures is due in part to the spread of the measuring current beyond the impedance electrodes which gives a similar foot in Fig 7, but it seems fairly certain that there is



FIG 11 The first derivative action potential  $V_i$ , vertical, against the bridge unbalance at 20 kc, horizontal during the passage of an impulse

<sup>8</sup> Cole K. S. and Curtis H. J. 1938-39, *J Gen Physiol* 22: 51

at least a measurable increase in conductance before the large and rapid increase takes place. The action potential corrections do not permit an accurate placement of this change, but it seems slightly to precede the reversal of the membrane current. This effect may be the impedance counterpart of the phenomenon preceding excitation at the cathode which has been observed by Katz (1937) and Hodgkin (1938).

The difficulties of determining the time of rise of the membrane conductance have already been discussed and the only conclusion to be made at present is that it is probably at least as short as 100  $\mu$ sec and perhaps even shorter. In contrast to the *Nitella* results, it will be noticed that for the squid axon the recovery of the action potential is completed considerably before that of the membrane resistance, but it seems likely that when this difference can be explained the whole phenomenon of excitation and conduction will be fairly well understood.

It should be possible, at this point, to determine the time course of the membrane electromotive force, by *Nitella* equation (16), and so obtain a complete picture of the electrical behavior of the membrane during activity. These calculations on *Nitella* were found to be very sensitive to small errors, so it seems best to avoid this procedure until other means have been exhausted and better squid data are available.

It seems apparent, however, from the data now available that, as was found for *Nitella*, the foot of the monophasic action potential up to the point of inflection represents a purely passive discharge into the active region following. Up to this point, the axon acts like a communication cable and although the conditions for the breakdown are being approached the axon has not yet exhibited any biological characteristics. At the point of inflection, we have the increase of conductance and decrease of electromotive force which give rise to the constant velocity and all-or-none behavior so characteristic of the propagated disturbance in excitable tissues.

The similarity of the impedance changes in the activity of *Nitella* and the squid axon is so striking as to add further proof that the phenomena of excitation and conduction are fundamentally the same in these forms. Since the action potentials of these forms are com-

parable with those of other nerve fibers and the membrane capacities of many cells, including the *Nitella* and the squid, cat sciatic, and frog sciatic axons, are nearly the same (*cf* Cole, 1939) we may assume for the present that there are impedance changes in other nerve fibers and that the mechanisms of excitation and propagation are all quite similar. In the future the impedance changes should be measured more accurately in *Nitella* and in squid and should be looked for in other forms, and the effects of various chemical and physical changes of environment should be investigated. There are a few preliminary observations on subthreshold phenomena and drug action but these are too incomplete to be discussed at the present time.

#### SUMMARY

Alternating current impedance measurements have been made over a wide frequency range on the giant axon from the stellar nerve of the squid, *Loligo pealii*, during the passage of a nerve impulse. The transverse impedance was measured between narrow electrodes on either side of the axon with a Wheatstone bridge having an amplifier and cathode ray oscillograph for detector. When the bridge was balanced, the resting axon gave a narrow line on the oscillograph screen as a sweep circuit moved the spot across. As an impulse passed between impedance electrodes after the axon had been stimulated at one end, the oscillograph line first broadened into a band, indicating a bridge unbalance, and then narrowed down to balance during recovery. From measurements made during the passage of the impulse and appropriate analysis, it was found that the membrane phase angle was unchanged, the membrane capacity decreased about 2 per cent, while the membrane conductance fell from a resting value of 1000 ohm  $\text{cm}^2$  to an average of 25 ohm  $\text{cm}^2$ .

The onset of the resistance change occurs somewhat after the start of the monophasic action potential, but coincides quite closely with the point of inflection on the rising phase, where the membrane current reverses in direction, corresponding to a decrease in the membrane electromotive force. This EMF and the conductance are closely associated properties of the membrane, and their sudden changes constitute, or are due to, the activity which is responsible for the all-or-none law and the initiation and propagation of the

nerve impulse These results correspond to those previously found for *Nitella* and lead us to expect similar phenomena in other nerve fibers

We wish to express our appreciation of the valuable assistance of Mr J M Spencer in all parts of this work

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# MEMBRANE AND PROTOPLASM RESISTANCE IN THE SQUID GIANT AXON

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## INTRODUCTION

The electrical structure of the squid giant axon has been investigated by measuring the alternating current impedance between electrodes placed on opposite sides of the axon. In these transverse measurements, the lines of current flow are primarily perpendicular to the axis of the fiber and the analysis of the results is relatively simple. It was found that the axoplasm is a good conductor and that it is bounded by a membrane with a relatively high resistance and a capacity of about  $1\mu\text{f}/\text{cm}^2$  (Curtis and Cole, 1938). Further experiments on active axons showed that the action potential was associated with a transient decrease in the electrical impedance (Cole and Curtis, 1938*b*, 1939). The decrease was primarily due to a fall in the membrane resistance to about  $25\text{ ohm cm}^2$  at the height of activity while the membrane capacity was relatively little affected by excitation. These measurements gave no information about the resistance of the membrane in the resting state because the current flow through that resistance is always negligible. The resistance is so high and the electrodes are so close together that at low frequencies most of the current flows through the external medium. At high frequencies, the current penetrates the cell through the capacity of the membrane, and the resistance is again effectively short circuited.

However, the resting membrane resistance may be determined from direct current measurements with a length of axon between two electrodes which surround the fiber. In such an axial or longitudinal arrangement, the lines of current flow are primarily parallel to the

axis of the fiber except where they cross the membrane. When the electrodes are close together nearly all of the current will flow in the connective tissue sheath,<sup>1</sup> but as the interpolar distance is increased, a larger proportion of it will cross the membrane in the neighborhood of one electrode, flow through the axoplasm, and leave in the neighborhood of the other electrode. In the squid axon, when the electrodes are a centimeter apart, about 80 per cent of the current crosses and recrosses the membrane. Since so much of the current flows through the membrane resistance, we may calculate it, as well as the resistances of the connective tissue sheath and the axoplasm, from the relation between the measured direct current resistance and the electrode separation.

It should be possible to calculate the unknown resistances from measurements made with any length of fiber in contact with the electrodes, but the analysis is involved unless the electrodes are very short or very long. When the electrodes are very short the calculation becomes simple, but the experiment is rather difficult. The interpretation of the data is somewhat more complicated when very long electrodes are used, but the experimental procedure is so much simpler that this method has been chosen.

#### EXPERIMENTAL METHOD

Fig. 1 shows the electrode system employed. One end of the fiber was drawn up through a narrow opening into the wide tube A which was joined to a calomel electrode and filled with sea water. The other end hung through a layer of oil into a large volume of sea water which connected with a second calomel electrode. The resistance of the two electrode systems was 800 ohms. The interpolar length was determined by the distance between the two sea water interfaces and could be varied by raising the upper electrode in an adjustable stand. Increments of distance could be measured to 50  $\mu$  by means of a micrometer scale which was attached to the screw control on the adjustable stand. The principal advantages of this electrode system were, first, that the interpolar distance could be altered very rapidly without changing the contact resistance of either electrode, and second that there was no opportunity for evaporation or condensation of water to alter the resistance of the preparation.

Giant axons were obtained from the hindmost stellar nerve of *Loligo pealii*. Great care was taken to remove as much connective tissue as possible because it

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<sup>1</sup> This term is used to describe the layer of diffuse connective tissue and sea water which clings to a fiber in oil or air.

introduced irregularities in the resistance length curve. About 35 mm of axon were required since the interpolar distance had to be increased to 10–15 mm and 10 mm were kept in each electrode. The reasons for using such long stretches in the electrodes are, first, that the theoretical analysis is based on the assumption of an infinite polar stretch, second, that the properties of the membrane might be altered by the injury currents which spread for several millimeters from the cut end. The diameter of the fibers was measured at intervals of 5 mm with an

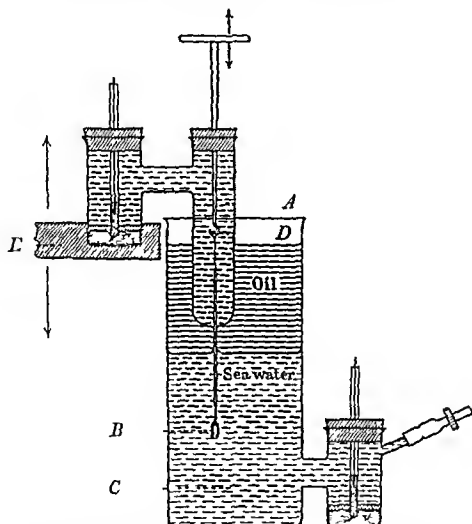


FIG. 1 Arrangement used for resistance-length determinations. E is an ebonite holder which is connected to an adjustable stand. For other letters see text.

eyepiece micrometer which magnified 70 times. Although most axons tapered slightly the diameter in the interpolar region was constant to within about 5 per cent and this variation did not cause any large error.

The method of mounting the fiber in the electrode chamber was as follows. After the dissection was complete a loop was tied in the silk ligature at one end of the fiber and a small platinum hook B attached to the other. The function of



this hook was to pull the fiber downwards and keep it in a vertical position when finally suspended in the electrode chamber. The upper electrode was raised out of the container C and lowered into a large dish of sea water. The fiber was placed in this dish and the silk loop at one end pushed through the 1.5 mm pore at the bottom of the tube A. This loop was caught in the glass hook D which was then pulled up until about 10 mm of fiber had been drawn into A. Finally the electrode and fiber were lifted out of the dish of sea water and lowered into position inside C.

At the beginning and end of each experiment the excitability of the whole axon was tested by recording the action potential with an amplifier and oscillograph. This was done inside the electrode chamber using the resistance electrodes as common stimulating and recording leads. The stimulus did not obscure the action potential, because it was applied through a balancing circuit. The resistance measurements were begun only if the axon was normally excitable. Some of the axons produced action potentials of 70 mv and survived for 6 hours in the electrode chamber.

The electrical resistance of the nerve was determined by applying a known voltage and measuring the current with a galvanometer (Leeds and Northrup, Type R,  $4.4 \times 10^{-10}$  amp/mm). The voltages were adjusted to produce a current of about  $0.4 \mu$  amp which was roughly one-tenth of that required to stimulate. The current was allowed to flow for about 15 seconds. Under these conditions the nerve seemed to behave as a simple resistance, since the current was proportional to the voltage and independent of its duration. An approximate value for the zero was first determined by noting the lowest resistance which could be obtained without permitting the two surfaces to coalesce. The resistance-length curve was usually started from a point which had a slightly greater resistance than this. Each observation was made with the current flowing first in one direction and then in the other. The two determinations usually agreed to within 3 per cent. The experiment was made as rapidly as possible in order to avoid progressive changes in the position of the zero and in the resistance of the nerve. When the curve was complete, one point was redetermined in order to make certain that there had been no serious drift in resistance. The experiments were usually made with increasing electrode separations, but occasional tests showed that similar curves could be obtained from measurements made in the reverse direction.

The experimental method was tested by measuring the relation between resistance and length in uniform rods of agar sea water. These were of the same dimensions as the nerve and could be mounted in the electrode chamber in a similar manner. The experimental points for the two rods, shown in Fig. 2, do not diverge from a straight line by more than 0.3 mm. As an additional test one experiment was made on a glass rod which had been coated with a thin layer of agar sea water. This also gave a linear relation between resistance and length.

Fig. 2D shows the resistance-length relation measured on a living axon with

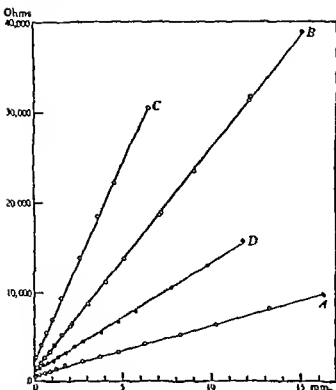


FIG 2 Resistance-length measurements on

A 720 $\mu$  agar rod

B 340 $\mu$  agar rod reinforced with 60 $\mu$  hair

C 670 $\mu$  glass rod coated with uniform layer of agar  $28 \pm 5\mu$  thick

D Living axon, resistance measured with 100 kc./sec alternating current

This axon was the same as that in Fig 3 and had a diameter of 540 $\mu$

Temperature 22-25°C Zero distance taken at point where two surfaces coalesce Zero resistance as electrode resistance when two surfaces have coalesced

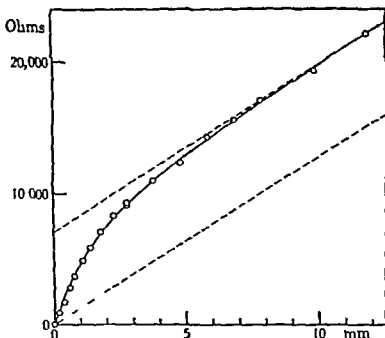


FIG 3 Relation between resistance and length on a 540 $\mu$  axon The upper dotted line is drawn asymptotic to the curve for large lengths the lower one through the origin and parallel to the upper line Observations made with increasing electrode separations upper point at 2.8 mm determined at end of experiment

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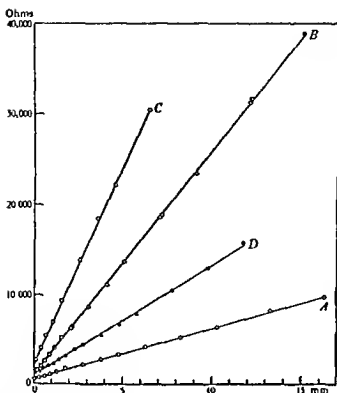


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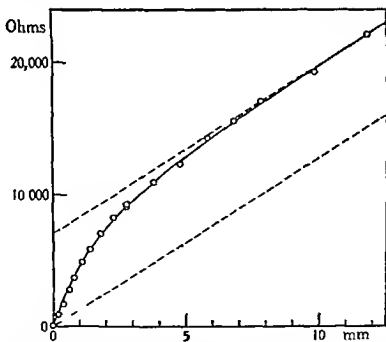


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100 kc alternating current by the method described by Cole and Curtis (1937). Since the nerve membrane has a low impedance to high frequency current, it cannot prevent an even distribution of current throughout the fiber. Hence the high frequency resistance is directly proportional to the interpolar distance and is determined by the parallel resistance of core and external fluid.

### *Experimental Results*

A typical resistance-length curve is shown in Fig. 3 where the circles are the experimental points. When the electrodes are close together very little current penetrates the fiber, because the resistance of the membrane is high compared to that of the external fluid. Therefore, the initial slope of the curve is steep and is determined by the external resistance. As the electrodes are separated and a greater area of membrane is exposed, more current penetrates the fiber. Hence the slope of the curve decreases, until at 8 to 10 mm the relation becomes a straight line with a gradient equal to the parallel resistance of the core and the external fluid.

The form of the resistance-length relation is derived in the theoretical section from the equations of Kelvin (1856) which govern the spread of current in cable-like systems. There have been many applications of this type of analysis to nerve, and among the more recent are those of Labes and Lullies (1932), Rushton (1934), and Cole and Curtis (1936). With the type of electrode employed we obtain the equation

$$R = \frac{r_1 r_2}{r_1 + r_2} s + \frac{2r_1^2 \lambda}{(r_1 + r_2)(\sqrt{(r_1 + r_2)/r_2} + \coth s/2\lambda)} \quad (1)$$

where  $R$  = total resistance of nerve,  $s$  = interpolar distance,  $r_1$  = external resistance per unit length,  $r_2$  = core resistance per unit length,  $\lambda = \sqrt{r_4/(r_1 + r_2)}$ , and  $r_4$  is the resistance of a unit length of membrane.

Close agreement between the theoretical and experimental curves can be obtained as is shown in Fig. 3 where the smooth curve is drawn according to this equation.

For reasons which will be discussed, the interpolar distance  $s$  and the resistance  $R$  were both assumed to be zero when the upper and lower menisci just fail to touch. The constants in equation (1)

were determined by the method to be outlined later. For this particular fiber the following values were obtained

$$r_1 = 61\,800 \text{ ohm/cm}$$

$$r_2 = 16\,180 \text{ ohm/cm}$$

$$r_4 = 4110 \text{ ohm cm}$$

The membrane resistance per sq cm  $R_4$  can be calculated from  $r_4$  if the radius  $a$  of the fiber is known. Thus  $R_4 = 2\pi ar_4$ . The specific resistance of the protoplasm  $R_2$  may be obtained from  $r_2$  by the equation,  $R_2 = \pi a_2^2$ . The conducting material outside the fiber

TABLE I

Experiments made at 22–25°C. Zero taken at point of coalescence. Extreme values given in cases where data did not allow exact analysis. Brackets in first column indicate that successive measurements were made on one fiber.

Axon diameter	Membrane resistance ( $R_4$ )	Specific resistance of protoplasm ( $R_2$ )	Thickness of external fluid ( $b$ )
<i>micra</i>	<i>ohm cm<sup>2</sup></i>	<i>ohm cm</i>	<i>micra</i>
500	1100	34	14
{ 480	500–800	25–23	23–20
{ —	500–600	27–26	18–17
{ 360	900	28	8
{ —	1000	27	7
{ 540	1000	38	28
{ —	700	37	20
{ —	600	37	16
480	400	24	21
{ 460	600	22	13
{ —	400–700	27–22	15
{ —	600	26	12

consisted of a thin layer of connective tissue and sea water. The thickness  $b$  of this layer can be calculated approximately, if it is assumed to have the same conductivity as sea water, by  $b = \sigma/2\pi ar_1$ , where  $\sigma$  is the specific resistance of the material outside the fiber. This equation holds as long as  $b$  is small compared to  $a$ . In the present work  $\sigma$  has been taken as 20.5 ohm cm, which is the resistance of Woods Hole sea water at 25°. The fiber of Fig. 3 had a radius of 70  $\mu$ , consequently

$$b \approx 19.6 \mu, R_2 = 37.1 \text{ ohm cm}, R_4 = 697 \text{ ohm cm}^2$$

Our results are summarized in Table I. The axons in these experiments were excitable and their thresholds remained constant while the measurements were in progress. Measurements were made on five more fibers, but the results were rejected. Two were found to be inexcitable at the end of the experiment. Two were excitable, but large differences in the resting potential and irregularities in the shape of the action potential showed that they were in poor condition. Inspection of the resistance-length curves obtained from these fibers showed that the membrane resistance must have been much lower than any in Table I. One experiment, made on an axon with a large amount of loose connective tissue, gave a very irregular resistance-length curve which could not be analyzed.

### *The External Resistance*

Table I indicates that the thickness of the layer of sea water and connective tissue which adhered to a fiber in oil varied from 7–28 $\mu$ . We did not try to check this result directly, but visual inspection left no doubt that the external layer was very thin compared to the axon diameter.

### *The Protoplasm Resistance*

The average value for the specific resistance of the protoplasm is 29 ohm cm, which is about 1.4 times that of sea water. This result is smaller than those obtained from measurements with alternating current and transverse electrodes. Curtis and Cole (1938) obtained values ranging from 1.5 to 6.9 times that of sea water and gave 4 as an average.

### *The Membrane Resistance*

The values of membrane resistance lie between 400 and 1100 ohm cm<sup>2</sup> with an average of about 700 ohm cm<sup>2</sup>.

### *The Characteristic Lengths*

The characteristic lengths in oil varied from 1.8 to 3.8 mm with an average value of 2.3 mm. In sea water, the characteristic lengths were between 5.0 and 9.3 mm. The average value was 6.0 mm.

### Theory

*The Resistance Length Relation*—The nerve fiber is assumed to behave like a uniform cable, with an external resistance,  $r_1$ , per unit length, due to adherent connective tissue and sea water, a core resistance,  $r_2$  per unit length, due to the axoplasm, and a membrane, or insulation, resistance of  $r_3$  per unit length. The corresponding sheath, core, and membrane currents,  $i_1$ ,  $i_2$ , and  $i_3$ , are to be considered positive in the direction of current flow. The resting electromotive force of the membrane may be ignored when we assume that it is the same everywhere and is not affected by current flow. Since, by symmetry, there is then no potential difference across the membrane at the center of the interpolar region, distance and the potentials,  $V_1$  of the sheath and  $V_2$  of the core, will be measured from this point.

We shall define the interpolar region as the length of axon,  $s$ , in which  $r_1$  is uniform, and the polar region as the length of axon where the outside potential,  $V_1$ , is constant,  $r_1$  is negligible, and  $i_1$  is zero.

By Ohm's law, we have

$$\frac{dV_1}{dx} = r_1 i_1 \quad (2) \quad \frac{dV_2}{dx} = r_2 i_2 \quad (3) \quad \text{and} \quad V_1 - V_2 = r_3 i_3 \quad (4),$$

and by continuity of current

$$\frac{di_1}{dx} = -\frac{di_2}{dx} = i_4 \quad (5) \quad \text{and} \quad i_1 + i_2 = i_0 \quad (6),$$

where  $i_0$  is the total current flow. From (2), (3), and (6),

$$\frac{d(V_1 - V_2)}{dx} = r_1 i_1 - r_2 i_2 = (r_1 + r_2) i_1 - r_2 i_0 \quad (7)$$

By differentiation of (7) and substitution of (4) and (5),

$$\frac{d^2(V_1 - V_2)}{dx^2} = \frac{r_1 + r_2}{r_3} (V_1 - V_2) \quad (8)$$

The solution of this differential equation is

$$V_1 - V_2 = A \sinh x/\lambda + B \cosh x/\lambda \quad (9)$$

where  $\lambda = \sqrt{r_3/(r_1 + r_2)}$  is the characteristic length. Since  $V_1 - V_2$



= 0 when  $v = 0$ , we have  $B = 0$ . Differentiating (9) and substituting in (7) gives

$$\frac{d(V_1 - V_2)}{dx} = \frac{A}{\lambda} \cosh \frac{x}{\lambda} = r_1 z_1 - r_2 z_2 \quad (10)$$

with this value for  $A$ , (9) becomes

$$V_1 - V_2 = (r_1 z_1 - r_2 z_2) \lambda \tanh x/\lambda \quad (11)$$

Integration of (2) and substitution of (7) gives

$$V_1 = \int_0^x \frac{dV_1}{dx} dx = r_1 \int_0^x z_1 dx = \frac{r_1 r_2}{r_1 + r_2} z_0 x + \frac{r_1}{r_1 + r_2} (V_1 - V_2) \quad (12)$$

Then from (6) and (11)

$$V_1 = \frac{r_1 r_2}{r_1 + r_2} \left( x + \frac{r_1}{r_2} \lambda \tanh \frac{x}{\lambda} \right) z_1 + \frac{r_1 r_2}{r_1 + r_2} \left( x - \lambda \tanh \frac{x}{\lambda} \right) z_2 \quad (13)$$

and similarly,

$$V_2 = \frac{r_1 r_2}{r_1 + r_2} \left( x - \lambda \tanh \frac{x}{\lambda} \right) z_1 + \frac{r_1 r_2}{r_1 + r_2} \left( x + \frac{r_2}{r_1} \lambda \tanh \frac{x}{\lambda} \right) z_2 \quad (14)$$

These equations completely describe the interpolar region, where  $r_1$  is constant, and the coefficients of  $z_1$  and  $z_2$  are called resistance coefficients. The quantities  $V_1$ ,  $V_2$ ,  $z_1$ , and  $z_2$  depend upon conditions at the end of this region and it is necessary to determine them before the measured resistance can be calculated.

If the electrode potential is  $V_0$ , then corresponding to (13) and (14) we have the equations

$$V_0 - V_1 = \beta_{11} z_1 + \beta_{12} z_2 \quad (15)$$

$$V_0 - V_2 = \beta_{12} z_1 + \beta_{22} z_2 \quad (16)$$

where the resistance coefficients  $\beta_{11}$ ,  $\beta_{12}$ , and  $\beta_{22}$  are to be determined. In the electrode region proper, where the outside potential is constant, we may apply (13) and (14) by remembering that  $r_1$  is negligible and  $z_1$  is zero, and obtain

$$V_0 - V'_1 = 0, \quad V_0 - V'_2 = \sqrt{r_2 r_4} z'_2 \tanh x/\lambda_p \quad (17)$$

where  $V'_1$  and  $V'_2$  are the potentials of the sheath and core at the boundary of the electrode,  $\lambda_p = \sqrt{r_4/r_2}$  is the polar characteristic length, and  $x$  is the distance from the boundary to the point where the

sheath and core are at the same potential. We may expect these potentials to be equal where the axon is ligated, but if  $x = 2\lambda$ , we have within 5 per cent

$$V_1 - V'_1 = \sqrt{r_2 r_1} z_2 \quad (18)$$

The simplest and ideal condition is the one in which there is a sharp boundary between the polar and interpolar regions. Then  $V_1 = V'_1 = V_0$ ,  $V_2 = V'_2$ ,  $z_1 = z'_1$ , and by eliminating  $V$ ,  $z_1$ , and  $z_2$  between equations (6), (13), (14), and (18) we obtain for the resistance

$$R = \frac{2V_0}{i_0} = \frac{r_1 r_2}{r_1 + r_2} s + \frac{2r_1^2 \lambda}{(r_1 + r_2)(\sqrt{(r_1 + r_2)/r_2} + \coth s/2\lambda)} \quad (1)$$

*Analysis of Resistance Length Curves*—One way of finding the correct values for the three constants in equation (1) would be to try a number of values and select those which gave the best agreement between the theoretical and the experimental curves. This method would be possible if a single constant were involved, but it becomes exceedingly laborious when three have to be determined. The following method was finally adopted.

Equation (1) can be written

$$R = ms + y \quad (19)$$

where

$$m = \frac{r_1 r_2}{r_1 + r_2} \quad (20)$$

$$y = \frac{2r_1^2 \lambda}{(r_1 + r_2)(\sqrt{(r_1 + r_2)/r_2} + \coth s/2\lambda)} \quad (21)$$

When  $s$  is infinite we have

$$y_\infty = \frac{2r_1^2 \lambda}{(r_1 + r_2)(\sqrt{(r_1 + r_2)/r_2} + 1)} \quad (22)$$

Introduce a new constant  $h$  defined by the relation

$$(h + 1)^2 = \frac{r_1 + r_2}{r_2} \quad (23)$$

Then from (21) and (22) we have

$$\frac{y}{y_\infty} = \frac{2 + h}{1 + h + \coth s/2\lambda} \quad (24)$$

Equation (22) may be written

$$y_{\infty} = 2mh\lambda \quad (25)$$

where  $m$  and  $h$  are defined by (20) and (23)

Hence

$$\lambda = y_{\infty}/2mh \quad (26)$$

Substituting this value in (22) we obtain

$$\frac{y}{y_{\infty}} = \frac{2 + h}{1 + h + \coth h(ms/y_{\infty})} \quad (27)$$

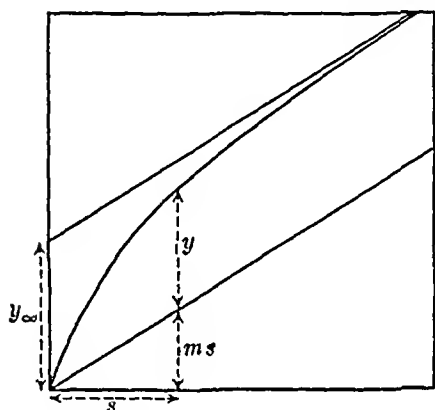


FIG 4 Diagram illustrating measurement of  $y$ ,  $y_{\infty}$ , and  $m$

$y$ ,  $y_{\infty}$ , and  $m$  can be measured directly from the resistance-length curve, as shown in Fig 4. Hence we can plot  $y/y_{\infty}$  against  $ms/y_{\infty}$  and obtain a curve which depends only upon  $h$ . The best value for  $h$  may then be determined by comparing this curve with a standard family of the form

$$\frac{2 + h}{1 + h + \coth h\lambda}$$

The values for  $r_1$  and  $r_2$  can be obtained by means of the following equations which may be derived from (20) and (23)

$$r_1 = m(h + 1)^2 \quad (28), \quad r_2 = \frac{m(h + 1)^2}{h^2 + 2h} \quad (29)$$

$\lambda$  can be determined from (26), and  $r_4$  from the relation,

$$r_4 = \lambda^2(r_1 + r_2) \quad (30)$$

#### DISCUSSION

*The Polar Region*—Equation (1) assumes an infinite length of axon in each electrode, but in practice there might be as little as 1 cm between a ligature and the interface. It is found in equation (18) that the polar resistance for an infinite stretch is  $\sqrt{r_2 r_4}$ . For a short-circuited end we have  $\sqrt{r_2 r_4} \tanh x/\lambda_p$  (17), and for an insulated end,

$\sqrt{r_1 r_2} \coth x/\lambda_p$ . In either case, when  $x > 2\lambda_p$ , the error will be less than 5 per cent of the polar resistance and a considerably smaller percentage of the total resistance. Since the average value of  $\lambda_p$  is 0.6 cm, the polar length should have been greater than 1.2 cm on this basis, but 1.0 cm was satisfactory. If the membrane is injured at the ligature in such a manner as to reduce the membrane resistance and approach the short circuited end condition, the experimental points should fall below the theoretical curve as the end nears the interface. However, it was found in every case that the resistance was higher than predicted, indicating that the resistance at and near the ligature was high and that the membrane was not severely injured in this region.

*The Meniscus Region*—The principal difficulty in the interpretation of the experimental data was caused by the capillary rise of the sea water on the axon at each oil-water interface. Because of it there is no simple way of determining either the electrode separation or resistance corresponding to a zero interpolar length, and these quantities should be determined accurately because the characteristic length in oil is quite small—2.3 mm.

In the theoretical section, the interpolar region was defined as the length of axon having a constant external resistance  $r_1$  and the polar region as that in which the sheath potential is constant and the longitudinal current  $i_1$  and resistance  $r_1$  are negligible, and these two regions were assumed to have a common boundary. This condition would probably be met if it were possible to pull each end of the axon into a thin close-fitting metal tube which would serve as an electrode, but with liquid electrodes there is certainly a transitional region.

As an approximation, the transitional region may be ignored and the resistance-length curve extrapolated back to a zero determined by the resistance of the electrodes alone. When this is done for the data in Fig. 3, the point of coalescence corresponds to an interpolar distance of about 0.4 mm and a resistance of 2100 ohms. From the analysis of the data on this basis,  $r_1$  is 20 per cent larger,  $r_2$  is 4 per cent less, and  $r_4$  is 25 per cent larger than was obtained above. This method of analysis was not generally employed because it has slight theoretical foundation and because the experimental points agreed

more closely with equation (10) when the point of coalescence was taken as the zero interpolar distance

If there were no meniscus and the sea-water oil interfaces were perfectly plane, there would be a crowding of the lines of current flow before they enter the sheath, which would result in a potential gradient in the sea water along the sheath and give rise to an external resistance. An approximate calculation of this resistance for the axon of Fig 3 gives a value of 1000 ohms for the two interfaces. The actual resistance would be higher than this because the interfaces were curved and not plane as assumed in the calculation. The smallest resistance observed experimentally was only about 2000 ohms, and so must have been due primarily to the constriction of the lines of current flow in the two menisci. Hence there is some justification for taking the point of coalescence as zero distance.

To determine the effect of the meniscus on equation (1), the combined characteristics of the meniscus and polar region may be given in the form of equations (15) and (16). From these and equations (13) and (14) we find the resistance

$$R = \frac{r_1 r_2}{r_1 + r_2} s + \frac{2r_1^2 \lambda p^2 q^2}{(r_1 + r_2)(q\sqrt{(r_1 + r_2)/r_2} + \coth s/2\lambda)} + R_0 \quad (31)$$

where  $R_0$  is the resistance for zero interpolar length,  $q$  is a function of  $\beta_{11}/\beta_{22}$  and  $\beta_{12}/\beta_{22}$  and  $p$  involves these ratios and  $r_2/r_1$ . This equation is too complicated to use for a direct evaluation of  $r_1$ ,  $r_2$ , and  $r_4$  and the method of analysis used with equation (1) is not valid but the errors can be estimated. For rough calculation we may take  $\beta_{11} = 1000$  ohms,  $\beta_{12} = 20$  ohms, and  $\beta_{22} = 8000$  ohms from equation (18), and then for the data on p 677,  $p = 0.97$ ,  $q = 0.88$ . It is found that  $r_1$  should be about 25 per cent greater,  $r_2$  about 7 per cent less, and  $r_4$  about 20 per cent less than the values previously calculated. The experimental points accidentally agree equally well with equations (1) and (31).

*The Membrane Resistance* — The method of analysis is based upon a theory which neglects the transitional region between the polar and interpolar lengths of the axon. It is best applied by measuring the resistance and interpolar length from their values when the upper and lower menisci just remain separate. A less satisfactory

method is to ignore the meniscus resistance and extrapolate the resistance length curve back to the resistance of the electrodes alone, this gives values for the membrane resistance which are 25 per cent larger than those obtained by the first method. A more complete theory including the transitional region is too involved for practical use, but approximations from it suggest a 20 per cent smaller membrane resistance.

The average value for the membrane resistance was 700 ohm  $\text{cm}^2$ , but we propose to adopt a value of 1000 ohm  $\text{cm}^2$  as a tentative estimate for the membrane resistance. Only a few of the axons had this membrane resistance, but they seemed to survive longer and so were probably more nearly normal. The membrane resistance may be high when the axons are in their natural state inside the body and may then fall to a relatively low value during the dissection. At present there is no way of deciding whether the value of 1000 ohm  $\text{cm}^2$  is a normal one, all that can be said is that it was obtained on axons which behaved as if they were in good physiological condition.

Previous measurements of membrane resistance have been made on *Nitella* and *Valonia*. Blinks (1930 b) obtained an average value of 250,000 ohm  $\text{cm}^2$  for the resistance of the thin protoplasmic layer which bounds the vacuolar sap in *Nitella*. However, *Nitella* is surrounded by fresh water, and for this reason its outer membrane may have a high resistance. Blinks (1930 a) found considerably lower resistances in the marine cell *Valonia*. Here the resistance varied from less than 1000 ohm  $\text{cm}^2$  in freshly gathered cells to over 50,000 ohm  $\text{cm}^2$  in cells which had been kept under constant conditions, and 10,000 ohm  $\text{cm}^2$  was given as an approximate value for a normal cell. In *Valonia* the current must cross two protoplasmic membranes, and the resistance of each would therefore be about 5000 ohm  $\text{cm}^2$  in a normal cell. The largest membrane resistance observed in our experiments was 1100 ohm  $\text{cm}^2$ . This suggests that the physiological condition of an isolated axon may be similar to that of freshly gathered *Valonia*.

The resistance length curves described in this paper are qualitatively similar to those obtained on medullated nerve by Rosenberg and Schnauder (1923), Lullies (1930), and Rushton (1934). Their experiments were made with whole nerve trunks and the results

cannot be analyzed with any certainty. However, a rough value for the combined resistance of myelin sheath and membrane can be obtained by making a number of simplifying assumptions as to the structure of the nerve trunk. Analysis of Rosenberg and Schnauder's data gives a value of  $4 \times 10^4$  ohm  $\text{cm}^2$  for the resistance of sheath and membrane. Hill (1932) calculated a value of  $2.2 \times 10^5$  ohm  $\text{cm}^2$  on the basis of Rushton's (1927) measurements. Both these estimates are open to a number of objections, but there can be little doubt that the combined myelin sheath and membrane resistance is much larger in medullated than in non-medullated fibers. This difference could be explained if myelin were a material with a high specific resistance.

#### SUMMARY

The direct current longitudinal resistance of the squid giant axon was measured as a function of the electrode separation. Large sea water electrodes were used and the inter-electrode length was immersed in oil. The slope of the resistance *vs* separation curve is large for a small electrode separation, but becomes smaller and finally constant as the separation is increased.

An analysis of the resistance *vs* length curves gives the following results. The nerve membrane has a resistance of about 1000 ohm  $\text{cm}^2$ . The protoplasm has a specific resistance of about 1.4 times that of sea water. The resistance of the connective tissue sheath outside the fiber corresponds to a layer of sea water about  $20\mu$  in thickness. The characteristic length for the axon is about 2.3 mm in oil and 6.0 mm in sea water.

We are indebted to Dr. W. A. H. Rushton for criticism, and to The Rockefeller Institute for Medical Research, New York, for the loan of apparatus.

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# THE EFFECTS OF ULTRAVIOLET RADIATION ON SPORES OF THE FUNGUS *ASPERGILLUS NIGER*

BY PAUL A. ZAHL, L. R. KOLLER, AND C. P. HASKINS

(From the Haskins Laboratory, Union College, and the Research Laboratory of the General Electric Company, Schenectady)

(Accepted for publication, March 16, 1939)

In the course of a series of studies now in progress concerning the effects of low velocity electrons upon the spores of highly standardized cultures of the Ascomycete fungus *Aspergillus niger*, it became of interest to undertake comparative work with ultraviolet light upon the same material. A large amount of work has been done upon the comparative killing effects of various wave lengths of ultraviolet upon unicellular organisms. Thus Ehrismann and Noethling (1932) and Oster (1934) have undertaken careful studies of the effects of equal intensities of ultraviolet of various wave lengths on the growth of yeast cells. Lacassagne (1930) and Wyckoff and Luyet (1931) have made extensive surveys of the types of cell injury which may supervene on the ultraviolet irradiation of yeast. Fulton and Coblenz (1929) have tested the validity of the Bunsen Roscoe relationship for intermittent ultraviolet irradiation of *Penicillium digitatum*. Nadson and Philippov (1928) and Hutchinson and Newton (1930) have reported stimulation in yeast under low dosages of ultraviolet, and Chavarría and Clark (1924) report similar stimulation for growth rate in cultures of *Monilomyces*. Schulze (1909) has reported a series of observations on the morphology of the mycelia of *Mucor stolonifer* irradiated with sublethal dosages of ultraviolet at 2900 Å. The production of giant cells has been observed by Elfving (1890), Reinhard (1923), and Lacassagne (1930) and Holweck (1932). Ramsey and Bailey (1930) have observed that conidia may be produced in a strain of *Fusarium* when exposed to ultraviolet which normally does not show such structures. The inhibitive effect of long exposures to ultraviolet upon spore production, and the apparently stimulative effect of short ones on *Mucor* were reported by Purvis and Warwick

(1907), while similar stimulative effects have been reported by Stevens (1928), Dillon-Weston (1932), Hutchinson and Ashton (1930), Ramsey and Bailey (1930), Bailey (1932), and Smith (1935). Bovie (1914, 1916) has reported the destructive action on germination with ultraviolet ranging from the longest wave lengths to less than 1700 Å. Smith (1935) has studied the effect of temperatures in conjunction with ultraviolet killing. Stevens (1928) has reported mutation under ultraviolet irradiation, in *Glomerella cingulata*, as has Dickson (1932) for *Chaetomium cochliodes*.

For the most part, the effects to be described are similar to those reported by one or more of these authors, but for several reasons the work is considered worthy of report at this time. The radiation sources available made it possible to obtain high intensities of practically monochromatic radiation. It is of interest to compare these ultraviolet results with those obtained with low voltage cathode rays, using the same cultures of material and the same culturing methods. Furthermore, certain unique qualitative results have been observed.

### *Source and Character of Radiation*

The source of radiation was a low pressure, hot cathode, mercury vapor discharge lamp in a glass having a high transmission for ultraviolet radiation. This lamp operates at a current of 0.25 ampere at 60 volts. Its output is practically monochromatic radiation of wave length 2537 Å. About 95 per cent of the energy radiated in the ultraviolet region lies in the 2537 Å line. At a distance of 1 meter from the lamp the intensity of this radiation is approximately 25 microwatts/cm<sup>2</sup>.

The intensity of radiation was measured by means of a sodium cathode phototube having a thin window of high transmission glass. Since this phototube has a long wave limit in the neighborhood of 4000 Å its readings could be used as a measure of the 2537 Å radiation. The tube was calibrated by Dr. B. T. Barnes of the Nela Park Laboratory of the General Electric Company.

### *Material and Methods*

The material used was exclusively the asexual spores of the Ascomycete fungus *Aspergillus niger*, the black bread-mold. These spores were obtained from cul-

tures of the fungus which have been bred in pure strain and highly standardized, and cultured on a standard potato-maltose agar for a number of years (1934). The spores are uniformly spherical, with a nucleus which, at the ages rayed, is spherical and centrally placed in the spores. The spores vary in diameter from 3-5  $\mu$ .

Spores of between 3 and 12 days of age were brushed from the culture dish onto cellophane strips, a fine camel's hair brush being used for this purpose. The cellophane strips, with the spore surface toward the light source, were then exposed to the radiation under the conditions described for each experiment. Within 6 hours after exposure they were "printed" as a monolayer of sparsely distributed spores, onto the moist standard potato-agar medium, and were cultured at constant temperatures for from 6 to 12 hours. At this time the agar surface was examined under the microscope and the living and dead spores were counted. Numerous objective fields were counted so as to get an accurate appraisal of killing effect for each printed area.

*Aspergillus* spores after ultraviolet irradiation do not germinate at a completely uniform rate. It was therefore necessary to set up an arbitrary criterion of survival. At the end of 6 hours of culturing some spores have swollen and sprouted, others do not achieve this condition until between 9 and 12 hours after printing. It was found that after about 10 hours all the spores which had at this time swollen in a characteristic manner would ultimately develop mycelia and give all other evidences of normal growth. Any that had not at this time swollen were found not to swell or germinate at any later period. They were arbitrarily construed as being dead. Therefore, spores were counted after 10 hours of culturing and were thus categorically classified as living or dead. Survival ratios on the basis of such counts were computed as the ratio between the percentage survival of the experimental spores and the percentage surviving in the controls (which accompanied each experiment).

## RESULTS

*1 Killing*—The survival ratios of *Aspergillus* spores exposed to monochromatic ultraviolet radiation of wave length 2537 Å as a function of total incident energy are presented in Fig. 1. The radiation intensity at the surface of the material was 88 microwatts/cm or 880 ergs/cm<sup>2</sup>/sec. In Fig. 1 are shown the results of three experiments, in which the total energy to which the spores were exposed was varied by exposing for various lengths of time between 3 and 51 minutes. The curves are of the sigmoid form usually observed. It is interesting to compare these results with those reported by Fulton and Coblentz (1929). They report 13.3 and 0.5 per cent survival after exposures of 1 and 4 minutes respectively to a quartz burner.

operating at 320 watts. From available data it is possible to make a rough estimate of the incident energy used in their experiments. The 1 minute exposure corresponds to about 846,000 ergs/cm<sup>2</sup> of 2537 Å radiation. In their experiments, however, there must have been considerable additional energy of both shorter and longer wave lengths.

2. *Effect of Relative Humidity upon Killing*—It was especially desired to determine whether the relative humidity of the atmosphere in which the spores were placed at the time of their irradiation was a factor in their resistance to ultraviolet radiation. Such a question is of considerable interest in connection with practical problems of ultraviolet fungicidal action. Three experimental groups of spores were therefore set up to be irradiated under identical conditions. One of these groups was treated under ordinary atmospheric conditions (relative humidity during irradiation being 67 per cent). A second group was kept for varying periods of time before irradiation in a closed moisture-saturated chamber with a cellophane top. This group was irradiated without removing the cellophane top. The transmission of the cellophane was measured by means of a phototube and the distance from the source adjusted so that the energy incident on the spores was the same as in the previous experiments. The results are presented in the curves of Fig. 2. The data as presented in Fig. 2 are in terms of survival ratios, and in this form show a striking similarity to the normal survival curves of Fig. 1. The absolute survival data (as distinguished from the ratio) of the desiccated spores, however, are about 20 per cent lower than those for the spores irradiated at atmospheric humidity. The absolute survival data of the "saturated" spores are approximately the same as those for the atmospheric spores, possibly a little lower, but not significantly so. The important observation, however, is that in terms of survival ratio the data from these aforementioned experiments fit into the same band as those from the atmospheric spores (*cf* Figs. 1 and 2), indicating that relative humidity is not of great importance in determining the fungicidal efficacy of 2537 Å irradiation.

3. *Killing As a Function of Ultraviolet Wave Length*—It is a matter of considerable interest, both theoretically and practically, to determine the relative efficiencies in killing of equal energies of ultraviolet of different wave lengths. Experiments were therefore under-

taken with four wave lengths of 3650 Å, 3129 Å, 3022 Å, and 2537 Å respectively, obtained by means of a monochromator. The results are plotted in Fig 3 where survival ratios are shown as a function of

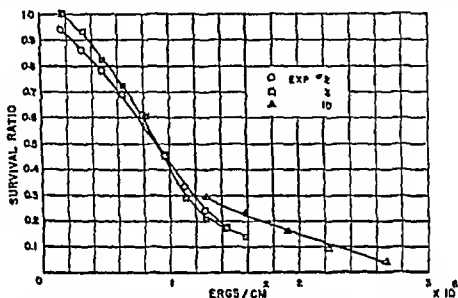


FIG 1 Survival ratio of *Aspergillus* spores exposed to 2537 Å radiation. Intensity 880 ergs/cm<sup>2</sup>/sec — 88 microwatts/cm<sup>2</sup>. Atmospheric conditions.

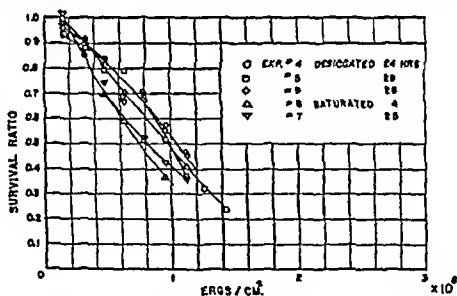


FIG 2 Survival ratio of *Aspergillus* spores exposed to 2537 Å radiation. Intensity 880 ergs/cm<sup>2</sup>/sec — 88 microwatts/cm<sup>2</sup>.

total incident energy for different wave lengths. The curve for wave length 2537 Å is repeated from Fig 1. These results show that the killing power falls off markedly with the increasing wave length and is negligible for wave length 3650 Å.



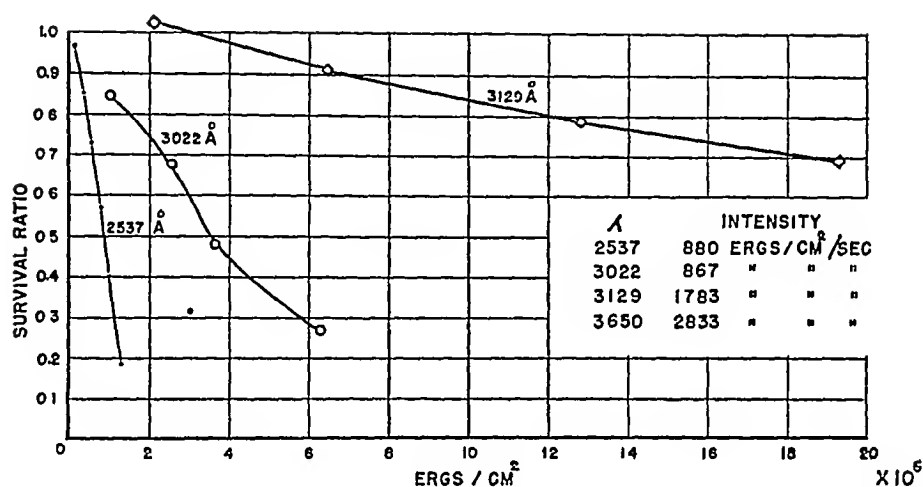


FIG 3 Survival ratio of *Aspergillus* spores exposed to radiation of various wave lengths

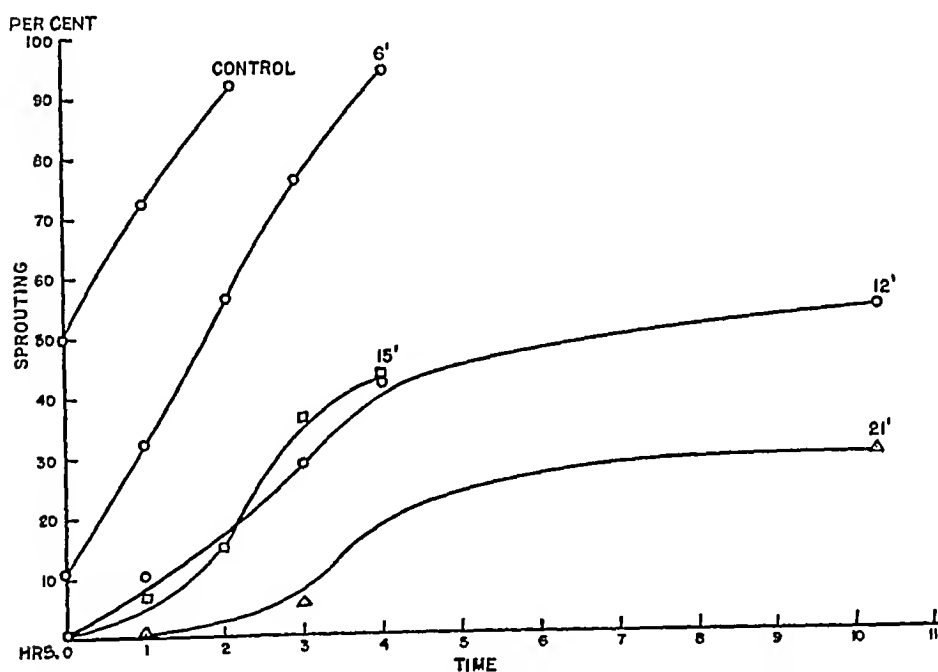


FIG 4 Effect of exposure time on germination of *Aspergillus* spores 2537 Å 88 microwatts/cm²

4 *Delay in Germination Time*—Associated with the phenomenon of complete inactivation of irradiated spores by ultraviolet is a very marked delay of germination. This delay is a very definite function of the total incident energy to which the spores have been exposed. Experiments to test this were undertaken using 2537 Å radiation from the sources already described, and a constant energy input. Experiments were made with exposure times of 6, 12, 15, and 21 minutes, corresponding to total energies of 317,000, 634,000, 792,000, 1,110,000 ergs/cm<sup>2</sup>, and the percentage of spores showing mycelial heaks was recorded at various intervals between 7 and 18 hours after planting. The results are shown in the curves of Fig 4. It will be seen that there is a marked increase in delay of total germination with exposure time. This effect is very sharply differentiated from radiation

TABLE I

Expt. No	Dosage/min	Exposure	Total energy	Survival	No spores counted
	ergs	min	ergs/cm <sup>2</sup>	per cent	
1	844,800	1	845,000	55.0	649
2	422,400	2		56.0	733
3	211,200	4	'	48.5	481
4	105,600	8		61.0	642
5	52,800	16	'	57.5	483

damage, inactivation, since even the very delayed spores, once germinated, thereafter developed morphologically normally, and for the most part grew at fairly normal rates.

5 *The Bunsen-Roscoe Reciprocity Law*—The question as to whether constant dosages of radiant energy produce equivalent biological effects in living organisms, regardless of how applied, is one which has been given very considerable attention in the past.

Experiments in this field were undertaken with ultraviolet. A constant energy input of 845,000 ergs/cm<sup>2</sup> was given several cultures in five experiments. In the first case, the entire dose was given in 1 minute. In the second, the intensity was reduced to 1/2 and the time doubled. In the third, the intensity was quartered and the time increased fourfold and so on, the total variation in intensity being over a range of 16 to 1. The results are shown in Table I. It will be seen

that there is no systematic deviation in these values. Moreover, they fall within the calculated probable error for the number of spores counted. Within the accuracy of the measurements and the counts, therefore, it may be concluded that, in this experiment, the validity of the Bunsen-Roscoe relationship may be assumed for ultraviolet light of 2537 Å wave length, over a distribution of time and energy input per unit of 16-fold.

*6 Morphological Changes*—No attempt was made to distinguish mutations, which have been reported a number of times with ultra-

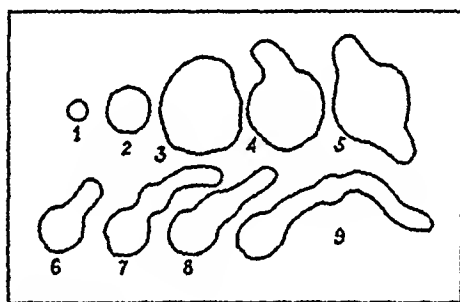


FIG 5 1 Normal *Aspergillus* spore, 3–5  $\mu$  in diameter

2 Normal swollen spore prior to sprouting

3 { Spores irradiated with 2537 Å ultraviolet while in the swollen condition  
4 { just prior to sprouting (2) Instead of sprouting normally the spores  
5 { undergo a continued swelling, reaching a diameter about three times normal  
before sprouting

6 Normal sprouting spore

7 { Spores irradiated during the early sprouting state (6) Continued growth  
8 { results in the bead-like structure on the mycelium  
9 {

violet, and only incidental attention was paid to morphological changes in the irradiated organisms. However, one change, similar to that recorded by other workers, was met with sufficiently frequently and was sufficiently conspicuous to deserve mention. Undue swelling of spores before germination under fairly heavy dosages given after swelling had begun—a phenomenon frequently reported by other workers for yeast cells—was noticed with considerable frequency. Many times germination was completely inhibited under these conditions, and the cells reached giant size without ever showing a more advanced condition. With mycelia already produced, however, when

irradiated, the mycelia formed large swellings or beads at their extremities. These persisted, even when the mycelium had developed beyond the enlargement. The enlargement was thus left like a bead on a thread. Occasionally several of these beads developed, and persisted throughout the period of observation of the spore. The various types of abnormalities most frequently observed are shown in Fig. 5.

#### SUMMARY

The survival ratio of *Aspergillus* spores exposed to ultraviolet radiation has been measured as a function of total incident energy for wave lengths of 2537 Å, 3022 Å, 3129 Å, and 3650 Å.

The effect of humidity on killing of *Aspergillus* spores by ultraviolet radiation has been found to be negligible.

A delay in germination as a result of irradiation has been found.

The Bunsen Roscoe reciprocity law has been found to hold within the limits of the radiation intensities studied.

Certain morphological changes have been observed.

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# INTRACELLULAR PHAGE PRECURSOR\*

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(Accepted for publication, May 3, 1939)

## INTRODUCTION

The early concept of phage production advanced by d'Herelle involved a general host parasite relationship between the bacterium and the homologous phage. The phage was thought to be an autonomous ultramicroscopic parasite which had the power to break through the cell's outer membrane, once inside the organism it divided into daughter corpuscles and the accumulation of new phage eventually resulted in cellular destruction or lysis. Certainly this description of the essential mechanism of bacteriophagy did fit the facts then available. However, the gradual accumulation of information concerning the reaction that takes place between the phage and bacterium has made it doubtful that this simple picture is correct.

It is now reasonably certain that phage is not an autonomous living agent, at least as judged by the criteria of classical physiology. The experimental evidence obtained by Krueger and his collaborators (1) led to the idea that phage is a protein with many of the properties of an enzyme (Krueger (2)), and Northrop (3) has supplied more direct proof of this concept by isolating a nucleoprotein which possesses all the characteristics of phage. This does not imply that phage occupies an entirely unique position in natural history as the only virus of its kind, for Stanley (4) sometime ago showed that tobacco mosaic virus consists of large protein molecules.

The demonstration of the fact that phage is a protein of high molecular weight assisted immeasurably in approaching the problem of its formation. Previous studies had indicated that bacterial lysis ensues when approximately 100 phage units per bacterium accumulate in

\* This work was supported by grants-in aid from the Research Corporation and from several interested friends

the system (1) Why bacterial growth was essential for the formation of phage did not appear. Later it was found that when bacteriophagy took place in the presence of 0.25 M NaCl (5) or 0.125 M Na<sub>2</sub>SO<sub>4</sub> (6) there occurred prelytic plateaus in the bacterial growth curves extending from 0.3 hour to 0.8 hour. Despite the fact that there was no bacterial reproduction during this time phage formation went on at a normal rate, suggesting that under certain conditions cell division was not requisite for phage production. Further evidence was supplied by the observation that the temperature and pH optima for the two rate curves differed (7).

These findings lead to the conclusion that cellular reproduction *per se* probably was not responsible for phage formation and suggested that some other cellular reaction operating optimally under nearly identical environmental conditions was accountable. The possibility that phage might be a product of bacterial metabolism had been stressed long ago by Bordet (8) and it now received support from the experiments of Krueger and Baldwin (9). They found that certain cell-free ultrafiltrates of normal staphylococcal suspensions when added to bacteriophage induced the formation of more phage in amounts well beyond the limit of error of the activity titration method. However, the phage-forming fraction appeared irregularly in culture ultrafiltrates and other means were sought for its demonstration.

Krueger and Mundell (10) have recently reported a method for demonstrating what they call "intracellular phage precursor." They observed that staphylococci grown in an oxygenated medium and subsequently maintained under conditions precluding cellular reproduction, had the capacity to increase [phage] very considerably when added to phage-containing solutions. This phage-augmenting characteristic of oxygenated or "activated" cells can be demonstrated with regularity and is not found in normal resting cells. The present paper deals with such properties of the intracellular "precursor" as we have been able to determine. In speaking of it as phage precursor we do so without knowing whether the essential phage-producing reaction is the hydrolytic cleavage of a preformed protein precursor, as seems to be the case with the autocatalytic transformation of inactive enzyme precursors into active enzymes, or whether it involves

completion of a complex protein synthesis by the cell under the stimulus of contact with phage

### EXPERIMENTAL RESULTS

Throughout the paper the following terms are used

- A. [Phage] = Concentration of phage/ml expressed as activity units
- B [Phage] = Initial concentration of phage/ml in activity units
- C P U = Phage units or activity units See Krueger (11) or Northrop (13) for discussion of the activity titration method.
- D [Bacteria] = Number of staphylococci/ml
- E [Bacteria] = Initial concentration of staphylococci/ml

*1 Demonstration of Intracellular Phage Precursor* —To demonstrate phage precursor in the strain of staphylococcus which has been used throughout all our previous work on bacteriophage the organisms are grown for 18 hours on nutrient agar in Blake flasks at 37°C The cells are washed in Locke's solution and are then grown in broth through which oxygen is constantly bubbled The bacteria are separated from the medium, resuspended in Locke's solution, and are kept for 2 hours at 5°C in order to inhibit any further cell division This suspension contains what we call activated bacteria To 4 ml of the activated suspension containing  $5 \times 10^8$  bacteria/ml is added 1 ml of phage diluted with Locke's solution to contain  $1 \times 10^9$  activity units/ml The mixture is kept for 5 minutes at 5°C and is then titrated for phage content by the activity method (11) The final [phage] as shown in Table I is found to be approximately  $2 \times 10^9$  activity units/ml, that of the control prepared with nonactivated bacteria from the original 18 hour agar culture is  $2 \times 10^8$  activity units/ml

Certain objections to this experiment at once present themselves and must be answered before the phenomenon can be considered valid For example, it is quite possible that the bacteria which have been activated by growth in the presence of oxygen have simply acquired an enhanced capacity for reproduction When added to phage for the purpose of demonstrating precursor they may go on growing and thus may continue to produce phage as a function of cellular repro



duction, *i.e.*, under the conditions described in an earlier study (12). Another possibility depends upon the fact that when the mixture of activated cells and phage is diluted for titration a certain number of activated bacteria will be present in the final titration mixtures. The activity method for phage titration depends upon ascertaining the time of lysis of a normal bacterial suspension to which dilutions of the phage-containing unknown are added. It is conceivable that the activated organisms constitute a significant proportion of the whole cell population and that they might selectively overgrow the bacteria added for titration purposes. If they grow at a faster rate they will reach the lytic endpoint more rapidly and will give the effect of an increased initial phage concentration.

TABLE I

*Outline of Experiment to Demonstrate the Phage-Augmenting Capacity of Activated Staphylococci*

- 
- |   |  |
|---|--|
| a | Staphylococci "activated" by growth in O <sub>2</sub> medium at 36° C 2 hrs  |
| b | Cells washed and resuspended in Locke's solution ( $5 \times 10^8$ bacteria/ml)  |
| c | Activated cell suspension kept at 5° C 2 hrs   |
| d | 4 ml activated suspension added to 1 ml phage in Locke's solution containing $1 \times 10^9$ P U/ml. Mixture kept 5 min at 5° C and titrated. $[\text{Phage}]_0 = 2 \times 10^8$<br>$[\text{Phage}]_{\text{final}} = 2 \times 10^9$  |
| e | Control 4 ml original 18 hr agar culture diluted in Locke's solution to contain $5 \times 10^8$ bacteria/ml added to 1 ml phage containing $1 \times 10^9$ P U/ml. Mixture kept 5 min at 5° C and titrated. $[\text{Phage}]_0 = 2 \times 10^8$ $[\text{Phage}]_{\text{final}} = 2 \times 10^8$ |
- 

These objections, we believe, have been answered by the following experiments

*A Activated Bacteria Carried over into Titration Mixture May Have an Untoward Effect on Accuracy of Results*

Standard phage containing  $1 \times 10^{10}$  activity units/ml is diluted in three different sets of broth blanks containing respectively  $5 \times 10^5$  activated bacteria/ml,  $5 \times 10^4$  activated bacteria/ml, and  $5 \times 10^3$  activated bacteria/ml. These concentrations of activated organisms represent the numbers which would be present when the mixture used to demonstrate the presence of precursor is diluted for titration. To 4 ml of each dilution of phage 1 ml of normal bacterial suspension ( $12.5 \times 10^7$ /ml) used for titration is added. The time of lysis of the

various mixtures is determined and it is found (Table II) that the small amounts of activated bacteria present in the mixtures do not affect the titration values in any way

*B Bacterial Growth May Occur in the Activated Bacteria Phage Mixtures and This May Be Responsible for the Observed Increase in Phage Titre*

As soon as the activated organisms prepared as described above were suspended in Locke's solution at 5°C samples were taken at successive intervals for direct microscopic counts and for plate counts. Samples were also taken as soon as the mixture of phage and organisms

TABLE II

*Outline of Experiment to Test Effect of Activated Bacteria Added to Phage Titration System*

- 
- 1 9 ml broth blanks prepared containing three different concentrations of activated bacteria
    - $5 \times 10^4$  activated bacteria/ml
    - $5 \times 10^4$  activated bacteria/ml
    - $5 \times 10^4$  activated bacteria/ml
  - 2 Sets of each concentration used to dilute standard phage for titration (from  $1 \times 10^{10}$  activity units/ml to  $1 \times 10^1$ ,  $1 \times 10^2$  and  $1 \times 10^3$  activity units/ml)
  - 3 To 4 ml of each dilution of phage 1 ml of normal bacteria containing  $12.5 \times 10^7$  cells/ml was added.
  - 4 Time of lysis was determined
  - 5 Presence of  $5 \times 10^4$ ,  $5 \times 10^4$  or  $5 \times 10^4$  activated bacteria/ml in titration system does not affect results
- 

in Locke's solution had been made. Due to the presence of phage only direct microscopic counts could be done on these latter mixtures. However, no growth was observed in the suspension containing activated bacteria alone or in the mixture containing activated bacteria and phage (Table III). Both preparations were followed for a 2 hour interval.

*C If Phage Precursor Exists It Should Be Possible to Serially Dilute a Given Amount of Phage in Successive Aliquots of Precursor without Reducing the Original Phage Titre*

One way to accomplish this would be to add sufficient phage to the precursor containing organisms so that after the precursor had

been converted into phage it would be released by cellular lysis. The lytic threshold for this particular strain of staphylococcus is 100 activity units/bacterium. When enough phage is added to bring about lysis the phage-bacteria ratio is so high that the small increment of phage derived from the intracellular reaction is not detectable. It is possible to circumvent this difficulty by utilizing the fact that small concentrations of  $Mn^{++}$  ions greatly reduce the lytic threshold. We have reported elsewhere (14) experiments in which the serial production of phage from the intracellular precursor of manganese-

TABLE III

*Tests for Growth of Activated Bacteria. Average Values of Three Experiments*

Activated cells were diluted to  $5 \times 10^8$  bacteria/ml in Locke's solution and were stored at  $5^\circ C$ . At intervals samples were removed for direct microscopic counts, plate counts, and for an activation test conducted as described in Table I.

In addition, direct counts (last column) were made on a mixture containing 4 ml of activated cell suspension + 1 ml of phage diluted in Locke's solution to  $1 \times 10^9$  P U/ml and kept at  $5^\circ C$ .

Time of sampling	[Bacteria] in Locke's suspension activated cells Direct count/ml	[Bacteria] in Locke's suspension activated cells Plate count	Test for activation of cell suspension Cells mixed with phage and titrated [Phage] = $2 \times 10^8$ P U/ml	Direct count on mixture of 4 ml activated cells + 1 ml phage diluted in Locke's solution to contain $1 \times 10^9$ P U/ml
hrs				
0 0	$4.5 \times 10^8$	$4.10 \times 10^8$	$4.0 \times 10^9$	$3.81 \times 10^8$
0 5	$4.35 \times 10^8$	$4.0 \times 10^8$	$3.5 \times 10^9$	$4.02 \times 10^8$
1 0	$4.61 \times 10^8$	$4.23 \times 10^8$	$4.0 \times 10^9$	$3.92 \times 10^8$
1 5	$4.47 \times 10^8$	$4.06 \times 10^8$	$4.0 \times 10^9$	$3.69 \times 10^8$
2 0	$4.28 \times 10^8$	$3.98 \times 10^8$	$4.0 \times 10^9$	$3.73 \times 10^8$

treated cells was carried out in the absence of cellular growth (Table IV). As each aliquot of intracellular precursor was converted into more phage the bacteria lysed and released a measurable quantity of phage free in the medium. The new lysate was then diluted to the original titre, more activated bacteria were added, etc. By this means the original phage was diluted to more than 1/2,000,000 without any loss of [phage] as determined by both the activity and plaque count titration procedures.

*2 Duration of Activation under Conditions of the Experiment—* Suspensions of activated organisms in Locke's solution were kept at

TABLE IV  
Serial Production of Phage from Successive Aliquots of Precursor Containing Bacteria

	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5	Mixture 6
Initial [phage]	$6.3 \times 10^8$	$6.3 \times 10^8$	$6.3 \times 10^8$	$6.3 \times 10^8$	$6.3 \times 10^8$	$6.3 \times 10^8$
Initial [bacteria]	$1 \times 10^8$	$1 \times 10^8$	$1 \times 10^8$	$1 \times 10^8$	$1 \times 10^8$	$1 \times 10^8$
Final [phage]	$7.9 \times 10^9$	$9.5 \times 10^9$	$6.3 \times 10^9$	$7.6 \times 10^9$	$5.7 \times 10^9$	$6.3 \times 10^9$
Total phage $\times$ total dilution	$6.3 \times 10^8$	$7.87 \times 10^8$	$11.8 \times 10^8$	$11.8 \times 10^8$	$14.2 \times 10^8$	$12.8 \times 10^8$

5°C and samples were removed at intervals. Each sample was mixed with a known amount of phage and after standing at 5°C for 5 minutes was immediately diluted for titration (Table V)

It was found that the suspensions of activated organisms maintained their activity for 4 hours without change. After this time there was some variation in the different preparations tested. Occasionally, 24 hour samples gave a typical increase in phage titre when added to phage, other suspensions completely lost their activity in 24 hours.

3 *Rate of Reaction between Activated Bacteria and Phage*—Suspensions of activated organisms were added to phage and samples were removed for titration at brief intervals in order to determine the time required for the typical increase in phage titre. In all cases the full

TABLE V

*Duration of Activation of Staphylococci after Growth in Oxygenated Medium*

$5 \times 10^8$  activated cells/ml stored in Locke's solution pH 7.4 at 5°C. 4 ml samples removed at intervals and tested by adding to them 1 ml of phage containing  $1 \times 10^9$  P.U./ml. Mixture kept at 5°C for 5 min and then titrated.

Preparation No	Total phage formed when activated cells added to phage after storage for				
	0 hr	1 hr	2 hrs	4 hrs	24 hrs
1	$3 \times 10^9$	$3.1 \times 10^9$	$2.8 \times 10^9$	$3.3 \times 10^9$	$9.6 \times 10^8$
2	$2.2 \times 10^9$	$2.2 \times 10^9$	$2.2 \times 10^9$	$2.2 \times 10^9$	$3 \times 10^8$
3	$2.0 \times 10^9$	$2.0 \times 10^9$	$2.3 \times 10^9$	$2.0 \times 10^9$	$2.6 \times 10^8$
4	$3.1 \times 10^9$	$2 \times 10^9$	$2.8 \times 10^9$	$2.8 \times 10^9$	$2.0 \times 10^9$

increase in titre was observed after the phage had been in contact with the organisms for 1 to 2 minutes (Table VI)

4 *The Role of Broth and Oxygen in Activation of Bacteria*—In order to determine whether oxygen and the broth medium were essential for activation, three different sets of bacterial suspensions were prepared.

A Normal activation mixture with oxygen bubbled through broth suspension of bacteria

B Nitrogen instead of oxygen bubbled through broth suspension of bacteria

C Oxygen bubbled through Locke's suspension of bacteria

It was found that very little activation was produced by nitrogen

bubbled through broth suspensions of staphylococci, oxygen bubbled through Locke's solution suspensions of staphylococci produced no measurable activation (Table VII)

TABLE VI

*Rate of Phage Formation by Activated Staphylococci*

16 ml of a suspension of activated staphylococci in Locke's solution containing  $5 \times 10^8$  bacteria/ml were added to 4 ml of phage diluted in Locke's solution to contain  $1 \times 10^9$  P U/ml. Temperature  $5^\circ \text{C}$  [Phage] =  $2 \times 10^8$ . Samples were removed at intervals for titration of total [phage]

Time after mixing phage and bacteria	Preparation 1 [phage]	Preparation 2 [phage]	Preparation 3 [phage]
m n			
1	$4.3 \times 10^8$	$2.0 \times 10^9$	$3.4 \times 10^9$
2	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.7 \times 10^9$
3	$2.2 \times 10^9$	$2.4 \times 10^9$	$6.7 \times 10^9$
4	$2.2 \times 10^9$	$2.5 \times 10^9$	$6.9 \times 10^9$
5	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.6 \times 10^9$
10	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.6 \times 10^9$
20	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.7 \times 10^9$
30	$2.0 \times 10^9$	$2.5 \times 10^9$	$6.6 \times 10^9$

TABLE VII

*Oxygen and Broth in Activation of Staphylococci*

Regular activation procedure carried out as described in Table I (column b below). In columns (c) and (d) are listed respectively activation values obtained with  $\text{N}_2$  replacing  $\text{O}_2$  with Locke's solution replacing broth. Column (a) lists the values obtained with an untreated 18 hour agar culture suspended in Locke's solution.

Preparation No	[Phage] formed after mixing 1 ml of phage ( $1 \times 10^9$ P U/ml) with 4 ml bacterial suspensions prepared in the following ways ([Phage] in all cases $2 \times 10^8$ P U/ml)			
	(a) Bacterial control Locke's suspension 18 hrs. agar growth	(b) Growth in $\text{O}_2$ -broth cells resuspended in Locke's solution	(c) Growth in $\text{N}_2$ -broth cells resuspended in Locke's solution	(d) Growth in $\text{O}_2$ -Locke's solution cells resuspended in fresh Locke's solution
1	$2.3 \times 10^8$	$5 \times 10^9$	$5.9 \times 10^8$	$2.1 \times 10^9$
2	$1.8 \times 10^9$	$2.1 \times 10^9$	$3.2 \times 10^9$	$2.0 \times 10^9$
3	$1.9 \times 10^8$	$4.6 \times 10^8$	$2.5 \times 10^8$	$1.7 \times 10^9$

5 *Effect of pH during and after Activation of Bacteria*—The activation process was carried out in broth of various hydrogen ion concen-

trations ranging from pH 5 to pH 9. The organisms were then tested for activation by adding bacteriophage at pH 7.4. No very significant differences were observed over the range studied although the mixtures prepared from organisms activated on the acid side of neutrality produced slightly higher phage titres (Table VIII).

The effect of hydrogen and hydroxyl ions on suspensions activated at pH 7.4 was tested by adjusting the pH of aliquots to various values and holding the suspensions for 1 hour at 5°C. An aliquot of each

TABLE VIII

*Effect of pH on Activation*

Activation with O<sub>2</sub> carried out by standard procedure (Table I) except pH of broth adjusted with acid or base to various pH's. After activation bacteria centrifuged down and resuspended in Locke's solution pH 7.4. 4 ml of each bacterial suspension added to 1 ml phage diluted with Locke's solution to contain  $1 \times 10^9$  P.U./ml. Mixture kept at 5°C. 5 min. and titrated

$$[\text{Phage}]_0 = 2 \times 10^8 \text{ P.U./ml}$$

pH of activation	Experiment 1		Experiment 2		Experiment 3	
	$[\text{Phage}]_{\text{final}}$	$\Delta [\text{phage}]_{\text{final}} - [\text{phage}]_{\text{initial}}$	$[\text{Phage}]_{\text{final}}$	$\Delta [\text{phage}]_{\text{final}} - [\text{phage}]_{\text{initial}}$	$[\text{Phage}]_{\text{final}}$	$\Delta [\text{phage}]_{\text{final}} - [\text{phage}]_{\text{initial}}$
5.08	$4.8 \times 10^9$	$4.6 \times 10^9$	$4.2 \times 10^9$	$4.0 \times 10^9$	$2.9 \times 10^9$	$2.7 \times 10^9$
5.55	$4.6 \times 10^9$	$4.4 \times 10^9$	$4.2 \times 10^9$	$4.0 \times 10^9$	$2.8 \times 10^9$	$2.6 \times 10^9$
5.91	$4.8 \times 10^9$	$4.6 \times 10^9$	$3.8 \times 10^9$	$3.6 \times 10^9$	$2.9 \times 10^9$	$2.7 \times 10^9$
6.65	$6.0 \times 10^9$	$5.8 \times 10^9$	$3.1 \times 10^9$	$2.9 \times 10^9$	$1.5 \times 10^9$	$1.3 \times 10^9$
7.02	$6.0 \times 10^9$	$5.8 \times 10^9$	$3.0 \times 10^9$	$2.8 \times 10^9$	$2.0 \times 10^9$	$1.8 \times 10^9$
7.44	$4.1 \times 10^9$	$3.9 \times 10^9$	$3.0 \times 10^9$	$2.8 \times 10^9$	$2.1 \times 10^9$	$1.9 \times 10^9$
8.15	$4.1 \times 10^9$	$3.9 \times 10^9$	$2.8 \times 10^9$	$2.6 \times 10^9$	$1.8 \times 10^9$	$1.6 \times 10^9$
8.40	$3.4 \times 10^9$	$3.2 \times 10^9$	$2.3 \times 10^9$	$2.1 \times 10^9$	$1.8 \times 10^9$	$1.6 \times 10^9$
9.00	$3.6 \times 10^9$	$3.4 \times 10^9$	$1.9 \times 10^9$	$1.7 \times 10^9$	$1.4 \times 10^9$	$1.2 \times 10^9$

suspension was then added to phage and the mixture titrated. As noted in Table IX the alkaline mixtures titrated somewhat lower than those exposed to moderate H<sup>+</sup> ion concentrations.

6 *Inhibition of Precursor-Phage Reaction by Antiserum*—Rabbits were injected repeatedly with nonactivated live staphylococci and with activated staphylococci. The initial doses used were small and injections were given three times a week using constantly increasing numbers of organisms. The serum of these animals and that of normal rabbits was tested for antibodies active against the phage precursor.

All three sera produced no inactivation when mixed with phage and subsequently titrated. Likewise none of the sera had a demonstrable bactericidal effect on normal or activated bacteria. However, it was found that the antiserum against activated bacteria and that produced against normal living staphylococci when mixed with activated organisms prevented the increase in [phage] usually observed upon the addition of phage to the activated organisms. This inhibiting effect could be demonstrated up to a 1/20 dilution of both sera. Normal

TABLE IX

*Effect of Storage at Various pH's on Activated Cells*

Activation carried out by standard method (Table I). Cells centrifuged down and resuspended in broth of various pH's. Suspensions kept at 5° C 1 hr. 4 ml of each suspension added to 1 ml of phage diluted in Locke's solution to contain  $1 \times 10^8$  P.U./ml. Mixture kept at 5° C 5 min and titrated. [Phage] =  $2 \times 10^8$  P.U./ml.

pH of bacterial suspension for 1 hr after activation at pH 7.4	Experiment 1		Experiment 2	
	[Phage] <sub>final</sub>	$\Delta$ [phage] final [phage] initial [phage]	[Phage] <sub>final</sub>	$\Delta$ [phage] final [phage] initial [phage]
5.08	$3.5 \times 10^8$	$3.3 \times 10^8$	$4.2 \times 10^8$	$4.0 \times 10^8$
5.35	$3.7 \times 10^8$	$3.5 \times 10^8$	$4.2 \times 10^8$	$4.0 \times 10^8$
5.91	$3.7 \times 10^8$	$3.5 \times 10^8$	$4.0 \times 10^8$	$4.0 \times 10^8$
6.65	$3.1 \times 10^8$	$2.9 \times 10^8$	$3.7 \times 10^8$	$3.5 \times 10^8$
7.02	$2.0 \times 10^8$	$1.8 \times 10^8$	$3.7 \times 10^8$	$3.5 \times 10^8$
7.44	$1.9 \times 10^8$	$1.7 \times 10^8$	$3.7 \times 10^8$	$3.5 \times 10^8$
8.15	$1.9 \times 10^8$	$1.7 \times 10^8$	$3.2 \times 10^8$	$3.0 \times 10^8$
8.40	$1.8 \times 10^8$	$1.6 \times 10^8$	$3.2 \times 10^8$	$3.0 \times 10^8$
9.00	$1.8 \times 10^8$	$1.6 \times 10^8$	$2.1 \times 10^8$	$1.9 \times 10^8$

rabbit serum did not prevent the reaction between activated cells and phage (Table X).

It is known that antibacterial sera readily form deposits on the surface of homologous organisms and it may well be that this surface film prevents the access of phage to the precursor containing portions of the activated cells. The data cannot very well be interpreted for or against the existence of the precursor as a distinct antigenic component.

**7 Heat Inactivation of Phage Precursor**—In experiments reported elsewhere (15) the rate of heat inactivation of phage precursor was



determined at several different temperatures. It was necessary to carefully control the time and temperature during heat inactivation of intracellular precursor so that no bacterial deaths would occur. If any appreciable number of cells died during the experiment they would adsorb phage irreversibly (2) when the latter was added to the suspension as a test for presence of precursor. The adsorbed phage would not participate in the titration reaction and the result would be an artificially reduced end titre giving false evidence for the inactivation of phage precursor.

TABLE X

*Inhibition of the Precursor-Phage Reaction by Antiserum*

Antisera prepared by injecting rabbits with live activated and nonactivated staphylococci. 1 ml of serum dilution added to 1 ml activated bacteria ( $1 \times 10^9$  cells/ml in Locke's solution) pH 7.4. After 1 hr at  $5^\circ\text{C}$  each mixture was added to 1 ml of phage diluted in Locke's solution to  $1 \times 10^9$  P.U./ml. This was kept 5 min at  $5^\circ\text{C}$  and was promptly titrated. Control experiments showed that the three sera had no bactericidal properties under the conditions of the experiment nor did they have any direct action on phage alone.

Serum dilutions used	Activated bacteria treated with serum 2 ml bacterial suspension added to 1 ml phage ( $1 \times 10^9$ P.U./ml) [Phage] <sub>0</sub> in all cases $3.3 \times 10^8$ P.U./ml		
	Total [phage] formed from suspension treated with anti activated bacteria serum	Total [phage] formed from suspension treated with anti nonactivated bacteria serum	Total [phage] formed from suspension treated with normal rabbit serum
1/2	$2.3 \times 10^8$	$3.0 \times 10^8$	$2.9 \times 10^9$
1/10	$3.1 \times 10^8$	$3.8 \times 10^8$	$2.9 \times 10^9$
1/20	$3.1 \times 10^8$	$3.1 \times 10^8$	$3.8 \times 10^9$
1/40	$3.9 \times 10^9$	$3.8 \times 10^9$	$3.9 \times 10^9$
No serum	$3.9 \times 10^9$	$3.9 \times 10^9$	$3.9 \times 10^9$

The critical thermal increment for the heat inactivation reaction was found to be about 90,000. Since figures of this magnitude are uniquely characteristic of protein denaturation reactions in general it would seem likely either that phage precursor is a protein or that the synthetic system which produces precursor contains a protein as an essential component. However, this does not exclude another possibility, namely, that the high temperature coefficient applies to heat induced changes in some entirely separate protein constituent of the

cell which when denatured may alter permeability conditions with the result that phage no longer has access to the precursor

8 *Quantitative Relationships*—Various concentrations of activated staphylococci were made in Locke's solution. To 4 ml amounts of each bacterial suspension 1 ml aliquots of different phage concentrations were added. The phage was run into the bacterial suspension drop by drop with constant stirring. The time of mixing was 1 minute after which the mixture was stirred for another minute. After standing for 4 minutes at 5°C the preparations were immediately diluted for titration.

With [phage]<sub>0</sub> constant as [bacteria]<sub>0</sub> increases the total [phage] rises to a maximal limiting value beyond which no further yield of phage is obtained. When the initial concentrations of phage are low the maximal values are reached with relatively small numbers of bacteria, with greater [phage]<sub>0</sub> more bacteria are needed for the production of the maximal yield of phage. As would be expected with large initial phage concentrations and very small numbers of bacteria there is no detectable increase in [phage] concentration.

A detailed account of the quantitative relationships obtaining between activated cells and phage will be published elsewhere.

#### DISCUSSION

The approaches to the problem of bacteriophagy have been beset by constant differences of opinion regarding the interpretation of experimental facts as well as by difficulties in establishing the facts themselves. Probably more divergent views have been developed on the nature of phage and the mechanism of its formation than for any other point in the phenomenon. According to d'Herelle phage is an autonomous living parasite which invades the body of the bacterium and once inside reproduces, synthesizing its substance from material available within the cell. Not all investigators have accepted d'Herelle's concept, many have agreed with Bordet (8) that phage probably is a by product of cellular metabolism and this view was strengthened by experimental evidence linking phage production with bacterial growth (12). It is undoubtedly true that bacterial growth and phage production go hand in hand under ordinary conditions but it is equally true that the two reactions may be separated. Krueger

and Fong (7) have shown that the pH and temperature optima for cellular reproduction and phage formation differ, in the present paper the evidence for phage production by activated cells in the absence of bacterial growth is summarized Northrop also has obtained experimental results which can be interpreted in no other way (16)

If phage formation is an intrinsic part of the cell's metabolic activities a possible mechanism would entail the elaboration of an inactive precursor within the cell and its subsequent transformation into active phage An ideal proof of this mechanism would be the isolation of the precursor in cell-free solution and the experimental demonstration that the precursor-phage reaction could be carried out in the total absence of the mother cell Krueger and Baldwin (9) found that the addition of phage to ultrafiltrates of growing staphylococcus cultures resulted in a 100 per cent increase in phage titre However, the results were irregular and it was not possible to get promising amounts of the phage-forming material Later Krueger and Mundell (10) demonstrated that staphylococci grown in an oxygenated medium and subsequently stored at 5°C in Locke's solution would increase [phage] 500 per cent within 2 minutes after phage was added to the cells It was shown first, that the reaction took place in the complete absence of bacterial growth and second, that the increase in titre did not depend upon any anomalous influence on the titration system

The results with the activated staphylococci were interpreted in terms of the precursor theory but in the absence of decisive data no attempt was made to decide whether the precursor-phage reaction involved the hydrolytic cleavage of a complex protein pro-phage or whether it was concerned with the catalytic completion of a cellular synthesis Whatever may be the case experimental data show that cells which have been brought to a resting state by storage in Locke's solution at 5°C after having undergone a period of active metabolism contain some component which reacts rapidly with phage to form more phage Live resting cells that have not undergone the preparative period of increased metabolic activity do not increase [phage] when brought in contact with phage solutions The precursor theory is compatible with the following experimental facts

- 1 The reaction between activated cells and phage is completed

within 2 minutes after mixing the reactants. This, of course, does not rule out the possibility that the mechanism proceeds as d'Herelle pictured it but it is hard to see how a living parasite could bore its way into the interior of the cell, synthesize its own substance, and reproduce within such a short time.

2 The serial dilution experiment together with the control experiments cited in this paper obviate the objection that the reaction reported may involve anomalous effects on the titration system. In the serial dilution experiment it has been found feasible to mix relatively high concentrations of manganese treated bacteria with sufficient phage to cause lysis without growth. The manganous ion depresses the lytic threshold and permits the use of sufficient cells so that the phage formed by the reaction between the activated cells and the added phage furnishes a measurable increment in [phage]. The newly formed phage is set free by cellular lysis and the reaction may be carried on in series, diluting the phage each time and adding fresh aliquots of activated cells. In these experiments the time elapsing is not concerned with phage production which takes place within 2 minutes but rather with the lytic process.

3 Activated staphylococci can be deprived of their phage augmenting capacity by heat treatment without causing cell death. After 20 minutes at 45°C activated cells cease to enhance [phage] when added to phage, at 50°C 2½ minutes are required to bring about the same result. During the application of heat for the periods noted there are no significant numbers of cell deaths. The rates of heat inactivation follow the curve of a monomolecular reaction and show a very high temperature coefficient. The critical thermal increment is 90,000 and is of the order of magnitude characteristic of protein denaturation reactions in general. It is probable, therefore, that the phage augmenting fraction of the activated cells either is a protein or that it contains a protein. However, another interpretation is not excluded for it is conceivable that as the cells are heated some entirely unrelated protein is denatured and that its denaturation alters cellular permeability preventing the access of phage to the cell.

4 The quantitative relationships outlined above are compatible with the precursor theory providing two assumptions are made. (A) That each activated cell contains a certain amount of precursor and

(B) That the phage formed remains attached to the cells Under the conditions of our experiments only traces of phage can be demonstrated in the suspending fluid

Starting with a small amount of phage the addition of a moderate number of activated staphylococci produces a measurable increase in [phage] As [bacteria]<sub>0</sub> is increased the total phage formed rises, finally attaining a maximal limiting value beyond which the addition of more cells ceases to have an effect With higher values of [phage]<sub>0</sub> the same general result is forthcoming except that the limiting plateau is shifted to the region of higher initial bacterial concentrations With very high [phage]<sub>0</sub> the smaller concentrations of activated cells produce no detectable increase in [phage] because the phage formed is negligible compared to the phage added

The reaction occurring when phage is added to activated bacteria cells represents only one phase of the complex phenomenon of bacteriophagy It shows that a bacterial cell prepared by a period of active metabolism is capable of reacting very rapidly with phage to form more phage, it does not tell how the newly formed phage is released to react with more bacteria Strong evidence for d'Herelle's concept that cellular lysis is an essential part of phage formation has been advanced recently by Ellis and Delbruck (17) Working with a strain of *B. coli* and a *coli* phage they found that the curve of phage increase was not strictly logarithmic with time but consisted of a series of plateaus connected by rather sharp slopes According to their interpretation phage is produced within the cell and is set free by the lytic destruction of the organism, the plateaus of the phage production curve cover the phase of adsorption and phage production within the cell, the steep portions coincide with the setting-free of intracellular phage by lysis For determining [phage] in their experiments they used the plaque count method, a procedure which gives a measure of the number of infected centers but does not estimate the total phage (i.e., a single phage-containing bacterium will produce one plaque whether it contains 1 or 100 activity units) The curve of phage increase is therefore the resultant of three related but not identical reactions, namely, the adsorption of phage, the intracellular production of phage, and the lytic destruction of the infected cell It expresses the rate of infection of a bacterial popula-

tion, which rate is dependent upon the speed of phage adsorption, the speed of phage formation, and the time required for the infected cell to burst. Our present data indicate that the first two of these reactions take place very quickly under the conditions outlined. Although it is not possible to apply these findings directly to the process of bacteriophagy taking place under normal conditions, they are not incompatible with the results of Ellis and Delbrück. They supply no evidence for the mechanism of phage transference from an infected cell to an uninfected one.

#### EXPERIMENTAL METHODS

1 Both the staphylococcus and anti staphylococcus bacteriophage used are the ones described in previous papers (1). In preparing the staphylococcus suspensions for daily use the organisms were grown on nutrient agar made with infusion broth, 1 per cent neopeptone and containing 2.5 per cent agar. The incubation period was 18 hours at 37° C after which the growth was harvested in Locke's solution and was washed once in Locke's solution before using. The cell concentration was determined by the centrifuged sediment method (18). The broth used was standard beef infusion containing 1 per cent Difco neopeptone 0.5 per cent sodium chloride, and was adjusted to pH 7.4.

2 Phage titres were determined by the activity method of Krueger (2). The activity unit is the smallest amount of phage which will cause lysis when added to a certain number of susceptible cells under standard conditions. Our standard phage contains  $1 \times 10^{10}$  activity units/ml. In practice three successive tenfold dilutions of each unknown were titrated.

3 For the preparation of activated cell suspension the washed staphylococci were suspended in broth in a concentration of  $5 \times 10^8$  bacteria/ml. The broth suspension was placed in a glass container arranged so that oxygen could be bubbled through the broth while the mixture was being shaken in a water bath adjusted to 36° C. After 1 hour of shaking the cell suspension was diluted with an equal volume of broth and the oxygen treatment was continued for another hour at 36° C. The cells were then centrifuged down and were resuspended in Locke's solution at pH 7.4. The suspension was kept at 5° C for 2 hours before using.

4 In testing the serum prepared against nonactivated staphylococci and activated staphylococci 1 ml of each serum dilution was mixed with 1 ml of a suspension of activated organisms containing  $1 \times 10^8$  bacteria/ml in Locke's solution at pH 7.4. The mixtures were kept at 5° C for 1 hour after which 1 ml of phage diluted in Locke's solution to  $1 \times 10^8$  P.U./ml was added. The mixture was kept an additional 5 minutes at 5° C and was then titrated. Controls included the same procedure carried out with normal rabbit serum and

tests for antiphage and bactericidal activity. None of the sera showed any significant capacity to inactivate phage or to kill staphylococci.

### SUMMARY AND CONCLUSIONS

1 Staphylococci activated by rapid growth in the presence of excess  $O_2$  and subsequently brought to a resting state by storage in Locke's solution at  $5^\circ C$  produce a significant rise in [phage] when added to phage-containing solutions.

2 For satisfactory activation the staphylococci require a period of active growth in the presence of oxygen. Activation proceeds best on the acid side of neutrality although variation in pH from 5 to 9 has relatively little effect. Activated cells retain their phage-augmenting property for from 4 to 24 hours, and this property may be destroyed by heating the cells at temperatures which do not kill them. The critical thermal increment for heat inactivation is 90,000 suggesting that the reaction involves protein denaturation.

3 The reaction between activated cells and phage has the following characteristics:

A It is complete in 1 to 2 minutes after mixing the reactants.

B The increase in phage does not depend upon bacterial growth nor does it involve any untoward effect on the titration system.

C Serum prepared by injecting rabbits with normal live staphylococci or with activated staphylococci when mixed with activated cells before the addition of phage will prevent the customary increase in [phage].

4 The phage-producing reaction which follows the addition of activated cells to phage can be interpreted in terms of the precursor theory. It is likely that the precursor either is a protein or contains a protein as an essential component.

5 There is no way of deciding at present whether the reaction between phage and precursor represents the hydrolytic cleavage of a protein or whether it is the final step in a synthesis catalyzed by phage.

We wish to express our thanks to Robert Brown for helpful technical assistance.

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# TEMPERATURE ACTIVATION OF THE UREASE-UREA SYSTEM USING CRUDE AND CRYSTALLINE UREASE\*

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Many studies of the kinetics of enzyme action as a function of temperature have been made. The older work (for reference cf Haldane, 1930, Tauber, 1937) indicates that while the velocity of an enzyme catalyzed reaction increases with rise in temperature, up to the inactivation temperature of the enzyme, this change in rate is not an exponential function of the absolute temperature. It has been pointed out by Bodansky (1937), however, that in many cases the velocity constants for enzyme reactions have been improperly computed. Certain recent work, on the other hand, (Crozier, 1924, Craig, 1936, Sizer, 1937, 1938 *a, b*, Gould and Sizer, 1938) indicates that for the fat oxidase from *Lupinus albus*, for yeast invertase either partially purified or present in the living cells, and for the anaerobic dehydrogenase system of *E. coli*, the reaction velocity increases exponentially with temperature according to the Arrhenius equation

$$K = ze^{-\mu/RT}$$

where  $K$  is the rate of the reaction,  $z$  is a constant,  $e$  is 2.718,  $R$  is the gas constant,  $T$  is the absolute temperature, and  $\mu$  represents the energy of activation of the reaction in calories per gram molecule. The  $\mu$  value is constant over a wide range of temperature until an inactivation temperature is reached, it is characteristic of the specific enzyme used, but is independent of the substrate employed and is not influenced by the conditions under which the reaction is carried out (Sizer, 1937, 1938 *a*), and is the same whether the enzyme is active

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inside the cell or extracted from the cell (Sizer, 1938 b) A particular critical increment might not characterize a single enzyme under all environmental conditions, because the conditions for activation of participating groups of the enzyme may be altered in such ways that one or another of several characteristic values could be secured

No studies of the temperature activation of enzyme systems as a function of the purity of the enzyme have been made If it were demonstrated that the temperature characteristic of an enzyme system varied with the purity of the enzyme, it would no longer be possible to say that a single  $\mu$  value characterizes a given enzyme system In this investigation a study has been made of the relationship between enzyme purity and the temperature characteristic of the urease-urea system in the presence of a variety of salts, especially those active in oxidation-reduction phenomena The following urease preparations were used an aqueous extract of jack bean meal, commercial urease (Arlco), and crystalline urease prepared according to the methods of Sumner (1926, Sumner and Hand, 1928)

## EXPERIMENTAL

### *Enzyme Preparations*

(a) *Jack Bean Meal Urease*—30 gm Arlco jack bean meal were suspended in 0.068 M sulfite (0.6363 per cent  $\text{Na}_2\text{SO}_3$  and 0.1416 per cent  $\text{NaHSO}_3$ ) and filtered The filtrate was diluted with 0.068 M sulfite until a solution with the proper activity was obtained

(b) *Commercial Urease (Arlco)*—A stable glycerol suspension was prepared according to the method of Koch (1937) Other preparations of Arlco urease were suspensions (either after filtering or without filtering) in water or sulfite (0.068 M was always used) As will appear later, it made considerable difference in the case of the sulfite solution whether the dry urease powder was added to a sulfite solution directly, or to water to which sulfite was added subsequently

(c) *Purified Commercial Urease*—Arlco urease was purified according to the methods of Sumner for preparing crystalline urease, and was reprecipitated once, but the product did not appear crystalline

(d) *Crystalline Urease*—Sumner's method for preparing crystalline urease from jack bean meal was followed closely The meal was suspended in 32 per cent acetone and filtered in the refrigerator The crystals which separated overnight in the filtrate were centrifuged free of the fluid, washed with 32 per cent acetone, and centrifuged again The urease was recrystallized by dissolving the crystals in a small volume of water, the solution was centrifuged free of insoluble material, and made up to 32 per cent with acetone Finally, a few

drops of phosphate buffer, pH 6.0, were added slowly. The crystals which formed were separated by centrifugation. Five separate batches of crystalline urease were prepared. In preparing the last two, 300 gm of meal were used instead of the customary 100 gm. In the case of preparations 3 and 4, urease fractions which had not been recrystallized were used in addition to the fraction which was once recrystallized. Preparation 5 was not recrystallized.

The activities of the crystalline urease preparations were not as high as those recorded by Sumner (100,000 to 130,000 units) ranging from about 1,000 to 55,000 units per gram. The lower activities may be attributed to the fact that high urease activities are recorded only when extremely concentrated urease solutions are used, but in these experiments only dilute urease solutions were suitable for use. It is also very likely that slight traces of metal impurities may have caused a slight inactivation or denaturation of the urease.

### *Estimation of Urease Activity*

A solution was prepared which contained 3 per cent urea, 5.4 per cent  $\text{Na}_2\text{HPO}_4$ , and 4.25 per cent  $\text{KH}_2\text{PO}_4$  and preserved with a few drops of toluene. 5 ml urea phosphate were added to 5 ml. urease solution after both solutions were adapted to the temperature of the water bath. At successive intervals 1 ml samples were removed from the digest and added to 1 ml normal HCl. The solution was diluted to about 75 ml to which were added 10 ml Nessler's solution, and it was finally diluted to 100 ml. The solution was then compared in a colorimeter with a standard  $(\text{NH}_4)_2\text{SO}_4$  solution treated in a similar manner containing 0.4 mg nitrogen (only 0.2 mg when the urease was very inactive) and also containing urea and phosphate so that the colors to be matched would be similar. In order to compensate for the fact that the weight of digest in the pipette varies slightly with the temperature of the digest, the standard was also adjusted to the temperature of the water bath. Thus the weight of digest and of standard were comparable at all temperatures. The temperature of the water bath was controlled to  $\pm 0.05^\circ\text{C}$ .

The solution was buffered with phosphate to pH 7.0 with the result that the alkalinity did not increase by more than 0.1 pH unit during the reaction. The hydrolysis was usually terminated after 0.5 mg ammonia nitrogen had been liberated. At each temperature the ammonia nitrogen was measured at seven different time intervals which elapsed before the 0.5 mg ammonia nitrogen had been liberated.

Since both crude and crystalline urease decompose rather rapidly in water, a stabilizer was usually added. For this purpose a glycerol extract (*cf* Koch, 1937) was used for crude urease, and a mixture of  $\text{Na}_2\text{SO}_3$  and  $\text{NaHSO}_3$  for crystalline urease (Sumner and Dounce, 1937). When it was discovered that the stabilizer might play an active rôle in determining the effects of temperature upon the reaction, a number of other compounds were substituted, among these were KCN,  $\text{Na}_2\text{S}_2\text{O}_3$ ,  $\text{CaCl}_2$ , cystine, cysteine, HCl,  $\text{Na}_2\text{SO}_4$ ,  $\text{K}_3\text{Fe}(\text{CN})_6$ ,  $\text{K}_4\text{Fe}(\text{CN})_6$ ,  $\text{H}_2\text{O}_2$ ,  $\text{FeCl}_3$ , I, sodium iodoacetate,  $\text{Cu}_2\text{O}$  and quinone.

## RESULTS

The kinetics of urea hydrolysis catalyzed by urease have been analyzed by several workers (Van Slyke and Cullen, 1914, Lovgren, 1921), but there is no general agreement concerning the course followed by the reaction. Many believe that it is unimolecular for at least a part of the time. In this study determinations were made during only the first part of hydrolysis, since in most enzyme reactions this is the most significant part (Haldane, 1930, Nelson, 1933). For all the enzyme preparations both crude and crystalline, in the presence of a variety of salts, and at all temperatures, the rate of liberation of ammonia nitrogen did not vary with time (Fig. 1). This in general held true until 0.6–1.0 mg. nitrogen had been liberated. The rate of ammonia liberation remains constant for a greater or lesser proportion of the hydrolysis, depending upon the particular preparation used and upon the temperature, the constant rate is often of greater duration at higher temperatures. After the linear portion of the reaction the hydrolysis usually becomes retarded, although with one urease preparation an acceleration occurred.

Scrutiny of Fig. 1 indicates that there is no characteristic difference in the kinetics of urea deamination catalyzed by crude and by crystalline urease. All the experimental data were plotted in the manner illustrated and rate of hydrolysis was calculated from the slope of the straight line drawn through the plotted points. This proved a simple and accurate method of determining milligrams of ammonia nitrogen liberated per minute, and duplicate runs usually checked within 5 per cent. While the data might have been analyzed equally as well according to the unimolecular equation, the method used was much simpler.

*Temperature Activation of Crude Urease*—The data obtained at different temperatures on urea hydrolysis using a jack bean meal filtrate suspended in sulfite were plotted and rate of reaction expressed as milligrams  $\text{NH}_3$  nitrogen liberated per minute. When log rate is plotted against  $1/T$  (Fig. 2, curve 1) the points are best fitted by a straight line over the temperature range of 0 to 40°C. Above the latter the points fall off from the curve indicating temperature inactivation of the urease. The slope of the line corresponds to an energy of activation of 11,700 calories for the urease-urea system. A

solution of Arlco urease was prepared by dissolving 0.67 gm in 100 ml sulfite and filtering free from insoluble material. An analysis of

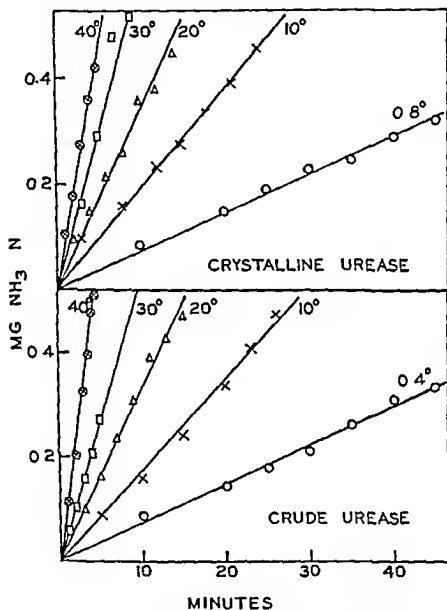


FIG 1 Hydrolysis (as measured by mg NH<sub>3</sub> N liberated) of 1.5 per cent urea in phosphate buffer, pH 7.0 catalyzed by urease dissolved in a sulfite solution is plotted as a function of elapsed time in minutes for several different temperatures

*Upper Curves*—Obtained using crystalline urease 2

*Lower Curves*—Obtained using the filtrate from a suspension of jack bean meal

the data on urea hydrolysis by this preparation yielded essentially similar results (Fig 2, curve 2). Strikingly different results were obtained, however, if the Arlco urease was dissolved in a glycerine

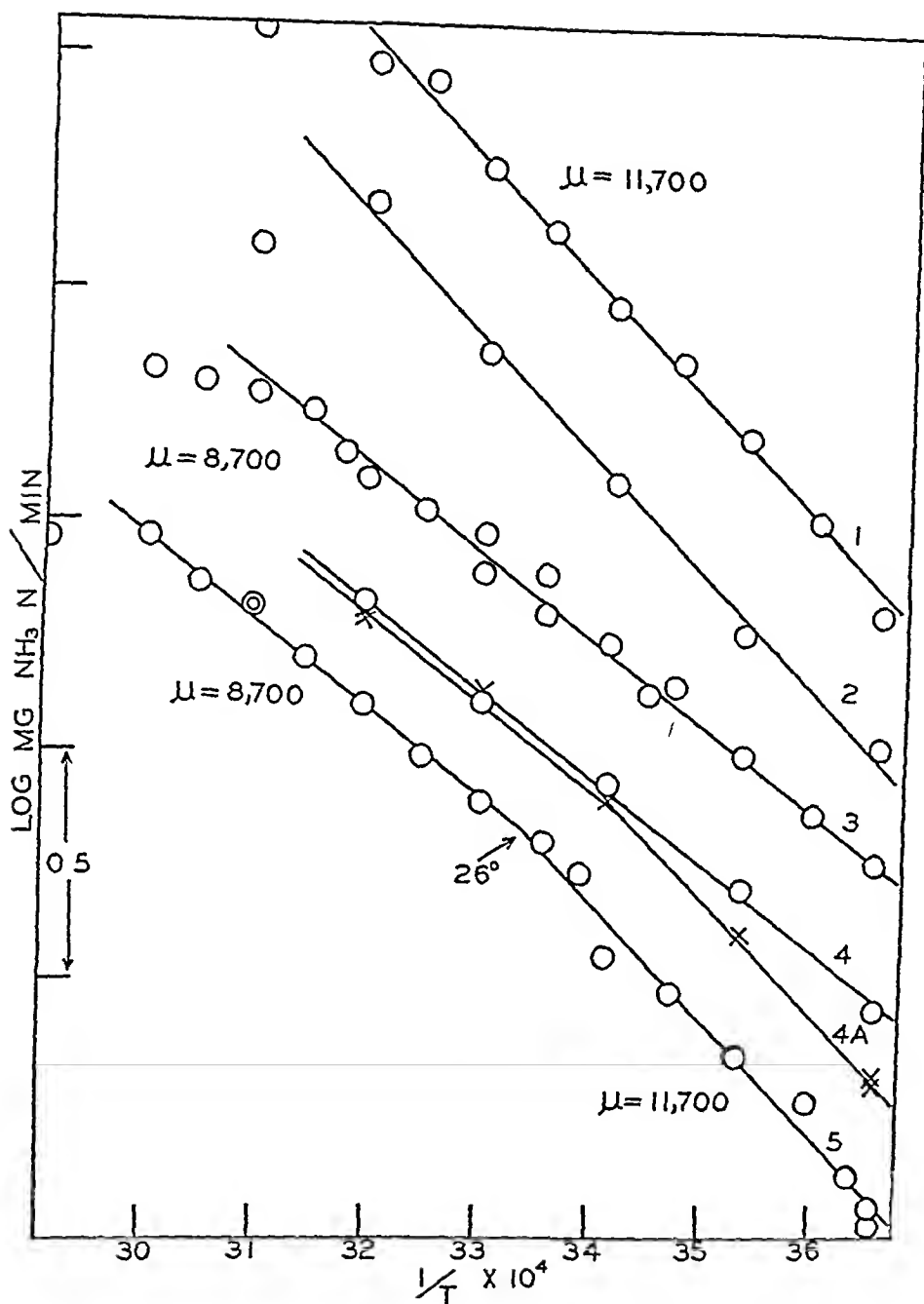


FIG 2 Log rate of NH<sub>3</sub> liberation from urea catalyzed by crude urease plotted against  $1/T$

- 1 Jack bean meal filtrate in sulfite solution,  $\mu = 11,700$
- 2 Arlco urease in sulfite solution,  $\mu = 11,700$
- 3 Arlco urease in glycerine solution,  $\mu = 8,700$
- 4 Arlco urease in water solution,  $\mu = 8,700$
- 4a Arlco urease in water + sulfite,  $\mu = 11,700, 8,700$
- 5 Purified Arlco urease in sulfite solution,  $\mu = 11,700, 8,700$

solution (cf Koch, 1937) (Fig 2, curve 3) or if 0.1 per cent Arlco urease was dissolved in water (Fig 2, curve 4). The slopes of the plotted curves are much less and both correspond to  $\mu = 8,700$  cal. When sulfite was added to the aqueous solution (Fig 2, curve 4a) the results were distinctly different from those obtained when Arlco urease was added *directly* to a sulfite solution (Fig 2, curve 2), or merely to an aqueous medium (Fig 2, curve 4). A break appears in the curve at  $25^{\circ}\text{C}$ ; over the temperature range of  $0$ – $25^{\circ}\text{C}$  the slope of the straight line which fits the plotted points corresponds to a  $\mu = 11,700$ , above  $25^{\circ}\text{C}$   $\mu = 8,700$ .

An attempt was made to prepare crystalline urease from 5 gm of Arlco urease by Sumner's method. The purified product consisted largely of denatured protein, had little activity, and showed no evidence of a crystalline nature when examined microscopically. The purified urease was dissolved in sulfite and its kinetics studied as a function of temperature (Fig 2, curve 5). An analysis of the data yielded results identical with those obtained with Arlco urease dissolved in water to which sulfite was added subsequently, namely a  $\mu = 11,700$  below  $26^{\circ}\text{C}$  and  $8,700$  above this temperature. Inactivation did not occur until  $65^{\circ}\text{C}$  was reached.

It is clear that with crude urease preparations an activation energy of  $8,700$  calories is obtained when the enzyme is dissolved in water or a glycerine solution. When dissolved in sulfite solution, however, the situation is more complex, under certain conditions a  $\mu$  value of  $11,700$  is obtained over the whole temperature range, while under other conditions the  $11,700$  value is obtained only below  $25$ – $26^{\circ}\text{C}$ , while above this critical temperature  $\mu = 8,700$ . It is surprising to observe that the purified urease was inactivated above  $65^{\circ}\text{C}$  while the crude jack bean meal urease lost its activity above  $40^{\circ}\text{C}$ .

#### *Temperature Activation of Crystalline Urease Dissolved in Sulfite Solution*

Crystalline urease preparations 1 and 2 were dissolved in sulfite solution and their kinetics studied as a function of temperature (Fig 3, curves 1 and 2). The results obtained by making an Arrhenius plot of the data on rate of hydrolysis of urea as a function of temperature were identical with those procured with highly purified Arlco



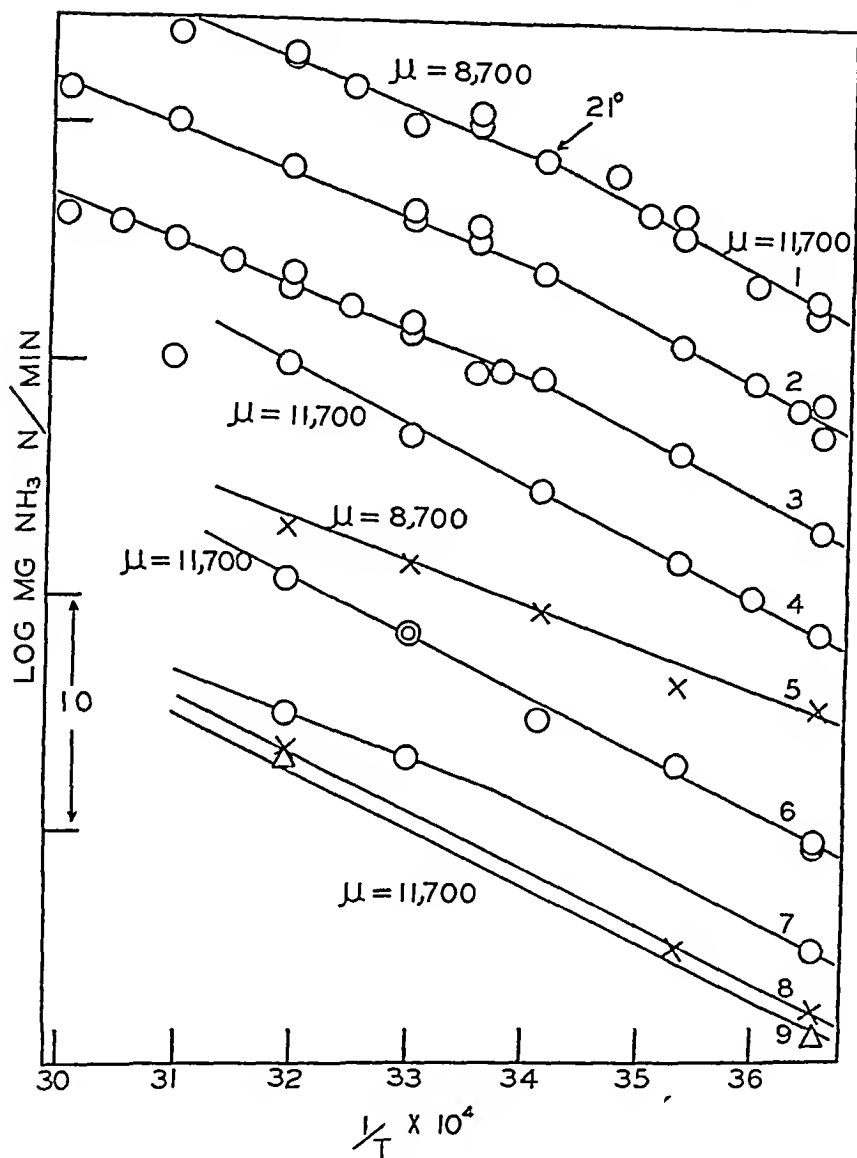


FIG 3 Log rate of  $\text{NH}_3$  formation catalyzed by crystalline urease dissolved in sulfite solution plotted against  $1/T$

- 1 Crystalline urease 1,  $\mu = 11,700, 8,700$
- 2 " " 2,  $\mu = 11,700, 8,700$
- 3 " " 3a,  $\mu = 11,700, 8,700$
- 4 " " 3,  $\mu = 11,700$
- 5 " " 3 (partially inactivated),  $\mu = 8,700$
- 6 " " 4a (dissolved first in water then  $\text{SO}_3$ )  $\mu = 11,700$
- 7 " " 4a in sulfite + 0.034 M  $\text{CaCl}_2$ ,  $\mu = 11,700, 8,700$
- 8,9 " " 4a in sulfite + excess  $\text{CaCl}_2$ ,  $\mu = 11,700$

urease or Arlco urease dissolved in water to which sulfite was subsequently added. Straight lines intersecting at  $21^{\circ}$  best fit the plotted points, below this critical temperature  $\mu = 11,700$ , above it is  $8,700$ . Anomalous results were obtained with the third preparation of crystalline urease dissolved in sulfite solution. A  $\mu = 11,700$  was obtained over the whole temperature range (Fig 3, curve 4). 5 days later when the preparation was used once more the urease activity was only  $\frac{1}{8}$  of the former value. While the data obtained on this partially inactivated enzyme were unreliable, there is little doubt that the  $\mu$  value has now completely changed to  $8,700$  (Fig 3, curve 5). The fraction of this same crystalline urease 3 which had not been recrystallized (called No 3a) behaved exactly like No 1 and No 2 with the usual two  $\mu$  values of  $11,700$  and  $8,700$  below and above  $22^{\circ}\text{C}$  (Fig 3, curve 3).

The studies on crystalline urease 4 in sulfite were made on that fraction which redissolved in a small volume of water but did not recrystallize from solution on adding acetone and phosphate buffer (this fraction is referred to as No 4a). A small sample of No 4a stock solution was diluted with sulfite and a  $\mu = 11,700$  was obtained over the whole temperature range (Fig 3, curve 6). Another sample in sulfite was made up to  $0.034\text{ M}$  with  $\text{CaCl}_2$ . A precipitate of  $\text{CaSO}_3$  formed. The solution now appeared to exhibit the double  $\mu$  situation, although the data are not very extensive (Fig 3, curve 7). The next day a  $\mu = 11,700$  prevailed over the whole temperature range (Fig 3, curve 8). An addition of more than enough  $\text{CaCl}_2$  to precipitate all the sulfite caused no further change in the solution (except that the activity fell somewhat), although it had stood for 2 days before being tested again (Fig 3, curve 9).

It appears from Fig 3 (curve 6) that if  $0.068\text{ M}$  sulfite solution is added to an aqueous solution of crystalline urease the preparation is characterized by a  $\mu = 11,700$ . A study was made to determine the effects of changing the sulfite concentration upon the temperature characteristic. The data obtained using crystalline urease 5 indicated that over the sulfite concentration range from  $0.0068$  to  $0.0306\text{ M}$   $\mu = 8,700$ , in the range from  $0.034$  to  $0.272\text{ M}$   $\mu = 11,700$ . A transition from one value to the other occurs at a sulfite concentration between  $0.0306$  and  $0.034\text{ M}$ .

In general, it may be stated that when crystalline urease is dissolved in a sulfite solution  $\mu$  values of either 11,700 or 8,700 prevail over the whole temperature range, or 11,700 below and 8,700 above the critical temperature of 21–23°C. Modifications in the urease solution may occur which effect a change from one activation energy to the other.

#### *Temperature Activation of Crystalline Urease Dissolved in Salt Solutions*

The preceding experiments on crude and crystalline urease suggest that the activation energy of the urease-urea system is determined by the configuration of the active groups in the enzyme molecule. It is well known that urease contains labile sulfur (Sumner and Poland, 1933) and that urease is readily inactivated (presumably by the oxidation of the SH to S-S) by oxidizing agents (Hellerman, Perkins, and Clark, 1933). As in the case of other enzymes of this type (*cf* Hellerman, 1937) if oxidation has not proceeded too far, the urease may be reactivated by the addition of reducing agents. Since the activity of crystalline urease is normally unaffected or only mildly increased by the addition of reducing agents, it appears that normally the urease is already in the reduced form. One interpretation of these experiments on the temperature activation of crude and crystalline urease is that when crude urease is dissolved in water or a glycerine solution, the urease active grouping is in a reduced state for which an activation energy of 8,700 is obtained, while when either crude or crystalline urease is dissolved in a sulfite solution, the enzyme may be in either a reduced form ( $\mu = 8,700$ ) or a partially oxidized form ( $\mu = 11,700$ ) depending on the temperature and other conditions. The change from one to the other is reversible, but never does urease behave kinetically as though both reduced and partially oxidized forms were active simultaneously at a single temperature and under the same conditions. This is evidenced by the fact that there is under no circumstance a blending of the two  $\mu$  values, the temperature characteristic is always either 8,700 or 11,700, never intermediate between the two.

In order to test the hypothesis that the activation energy is related to the configuration of certain active groups in the urease molecule, the urease medium was altered in a variety of ways calculated to stabilize urease in either its reduced ( $\mu = 8,700$ ) or partially oxidized form ( $\mu = 11,700$ ). In all these experiments crystalline urease 4

was used (fraction 4 *a* is the fraction which redissolved in a small portion of water, No 4 *b* the fraction which did not redissolve in a little water, No 4 *c* the fraction which was recrystallized once) The urease preparations were dissolved in a small volume of water Samples of these stock suspensions were diluted in salt solutions so that the final solution was 0.068 M in salt The following solutions completely inactivated the urease and their effects upon urease kinetics could not be measured  $\text{FeCl}_3$ , cysteine HCl,  $\text{Cu}_2\text{O}$  (saturated solution), quinone (saturated solution),  $\text{I}_2$  (saturated solution), sodium iodoacetate, and  $\text{H}_2\text{O}_2$  The inactivating action of the cysteine HCl may have been due to the unneutralized acid, of iodoacetate due to a trace of iodine present A 0.0137 M  $\text{H}_2\text{O}_2$  solution only partially inactivated the urease and so this preparation was used in a kinetic study

Crystalline urease 4 *a* dissolved in a KCN solution yielded a  $\mu = 8,700$  over the whole temperature range (Fig 4, curve 1) This same value was unchanged after an interval of several days Similar results were obtained with recrystallized urease 4 *c* (Fig 4, curve 2) In this case, however, the temperature characteristic was not stable and changed overnight to 11,700 (Fig 4, curve 3) A  $\mu$  value of 8,700 was also obtained over the whole temperature range when crystalline urease 4 *b* was dissolved in solutions of the actively reducing agents,  $\text{Na}_2\text{S}_2\text{O}_4$  (Fig 4, curve 4), and  $\text{K}_4\text{Fe}(\text{CN})_6$  (Fig 4, curve 8), the indifferent salt  $\text{Na}_2\text{SO}_4$  (Fig 4, curve 6), and the very weakly oxidizing reagent, cystine (Fig 4, curve 5) An attempt was also made to study the activity as a function of temperature when crystalline urease 4 *a* was dissolved in water This was very difficult since crystalline urease is extremely unstable in water (Sumner *et al*, 1938) By using three separate preparations, however, and studying hydrolysis at only two temperatures with each, it was possible to obtain data which clearly indicated a  $\mu = 8,700$  for urease dissolved in water (Fig 4, curve 7)

It appears that when dissolved in indifferent, actively reducing, or very mildly oxidizing solutions, crystalline urease is in a reduced form with an activation energy of 8,700 calories per gram mol of urease urea complex

Oxidizing agents were very difficult to use since most of them completely inactivated the crystalline urease Reliable data were ob

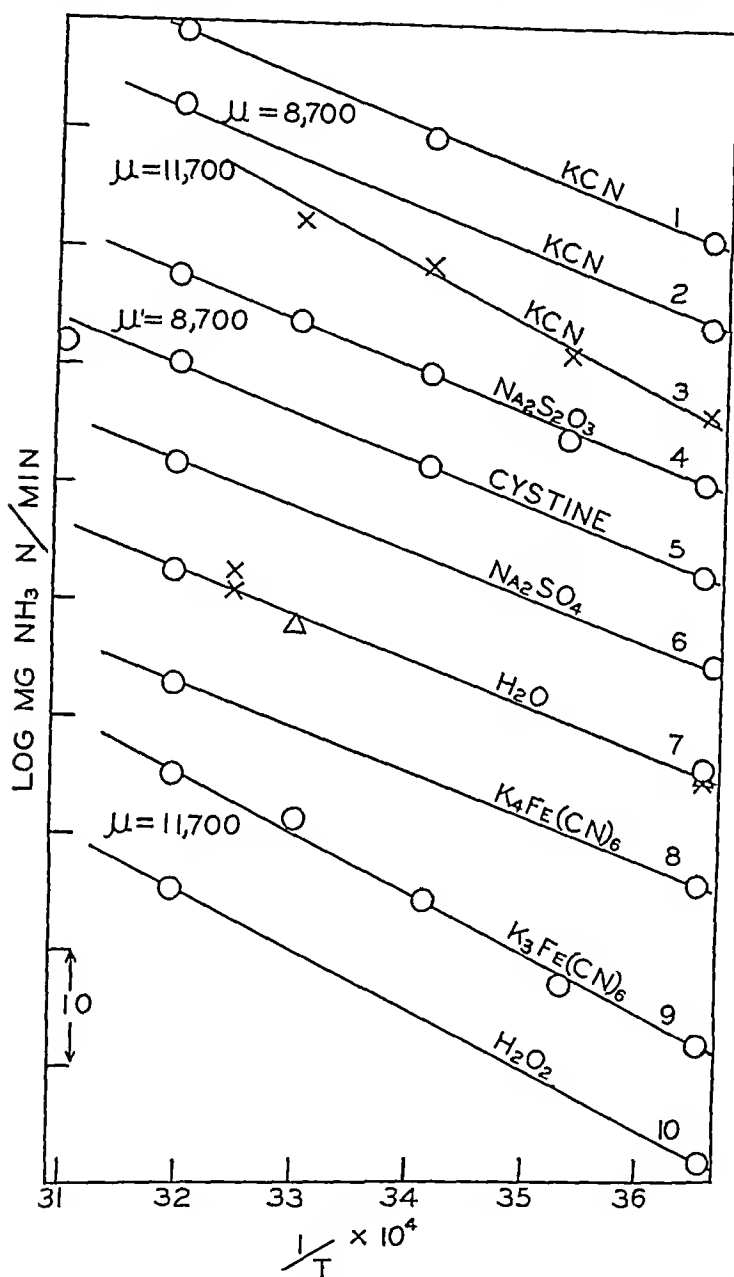


FIG 4 Log rate of  $\text{NH}_3$  formation catalyzed by crystalline urease 4 dissolved in a variety of solutions, plotted against  $1/T$ . When the enzyme is dissolved in water or solutions of KCN,  $\text{Na}_2\text{S}_2\text{O}_3$ , cystine,  $\text{Na}_2\text{SO}_4$ , or  $\text{K}_4\text{Fe}(\text{CN})_6$ ,  $\mu = 8,700$ . In one instance the enzyme dissolved in a KCN solution became slightly inactivated, and  $\mu$  changed from 8,700 to 11,700. When the enzyme was dissolved in  $\text{K}_3\text{Fe}(\text{CN})_6$  or  $\text{H}_2\text{O}_2$ ,  $\mu = 11,700$ .

tained, however, on crystalline urease 4 *b* dissolved in the active oxidizing solution of  $K_3Fe(CN)_6$  (Fig 4, curve 9) and of  $H_2O_2$  (Fig 4, curve 10) The analysis of the data suggests that when the urease is partly inactivated by oxidizing agents, the configuration of the urease molecule has undergone a change so that now the activation energy of the urea urease complex is 11,700 calories

The activity of crystalline urease was slightly greater in a sulfite solution than in water With most of the other reducing reagents the activity was about the same or slightly less than in water With the oxidizing solutions the urease activity fell to 1/3 ( $K_3Fe(CN)_6$ ) or 1/10 ( $H_2O_2$ ) of the original value These results are consistent with those obtained by others (Hellerman, 1937)

#### *Temperature Characteristics of Crystalline Urease As Related to the Redox Potential of the Solution*

From the previous sections it is evident that a  $\mu = 11,700$  is in general correlated with the presence of oxidizing agents in the urease solution, while a  $\mu = 8,700$  is associated with reducing or indifferent agents in the solution If the temperature characteristic of urease is determined by the redox potential of the enzyme solution and not by the type of electrolyte present, it should be possible to alter the  $\mu$  value by changing the oxidation reduction potential This was done by using crystalline urease 5 dissolved in varying mixtures of  $K_3Fe(CN)_6$  and  $K_4Fe(CN)_6$  In all cases the total salt concentration was 0.068 M

The results obtained are not as quantitative as could be desired because of the fact that urease is very unstable in  $K_3Fe(CN)_6$  solutions The rates of urea hydrolysis were calculated in the usual way and an Arrhenius plot made of the data (Fig 5) The distribution of the curves along the ordinate is purely arbitrary When the  $K_3Fe(CN)_6$  is three or more times as concentrated as  $K_4Fe(CN)_6$  a  $\mu = 11,700$  is obtained When the ratio of the two salts is the same or when one was only twice as concentrated as the other  $\mu = 11,700$  below and 8,700 above the critical temperature When  $K_4Fe(CN)_6$  is three or more times as concentrated as  $K_3Fe(CN)_6$   $\mu = 8,700$  over the whole temperature range

The oxidation reduction potentials of the urease urea solutions were measured with a Beckman pH meter at 26°C using a platinum elec-

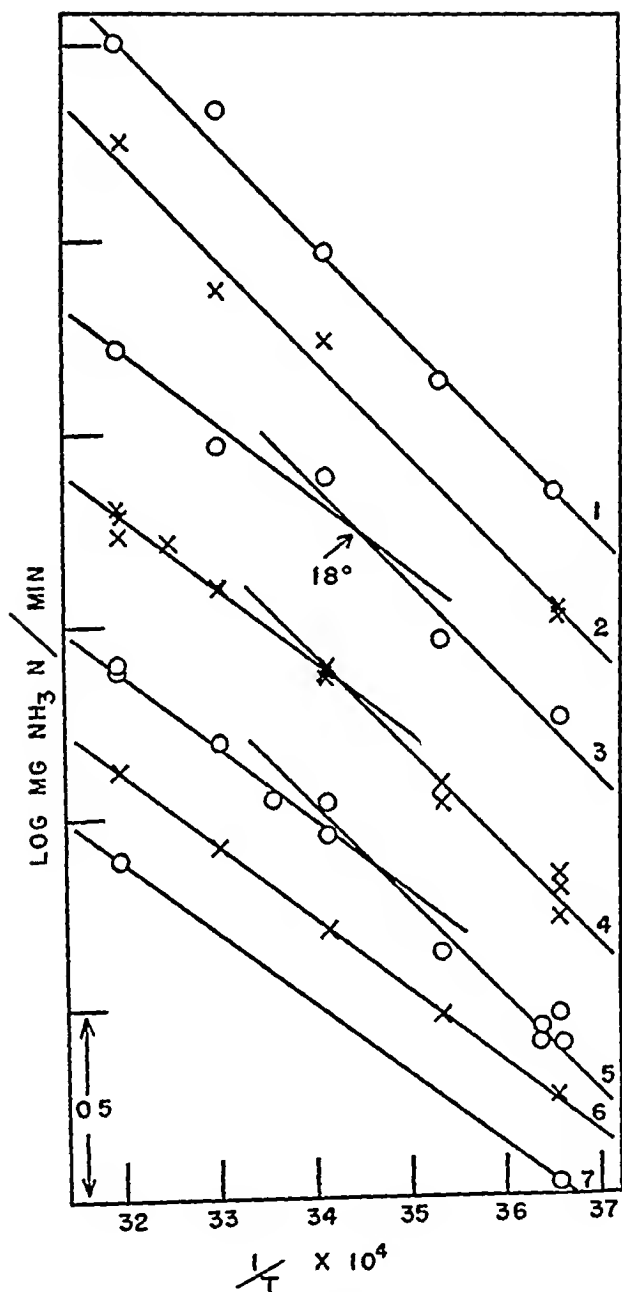


FIG 5 Log rate of  $\text{NH}_3$  liberation from urea catalyzed by crystalline urease dissolved in 0.068 M  $\text{K}_3$ - or  $\text{K}_4\text{Fe}(\text{CN})_6$  plotted against  $1/T$

- 1  $\text{K}_3\text{Fe}(\text{CN})_6$ ,  $E_h = +0.611$  volt,  $\mu = 11,700$
- 2  $\text{K}_3/\text{K}_4 = 3/1$ ,  $E_h = +0.474$  volt,  $\mu = 11,700$
- 3  $\text{K}_3/\text{K}_4 = 2/1$ ,  $E_h = +0.459$  volt,  $\mu = 11,700, 8,700$
- 4  $\text{K}_3/\text{K}_4 = 1$ ,  $E_h = +0.443$  volt,  $\mu = 11,700, 8,700$
- 5  $\text{K}_3/\text{K}_4 = 1/2$ ,  $E_h = +0.420$  volt,  $\mu = 11,700, 8,700$
- 6  $\text{K}_3/\text{K}_4 = 1/3$ ,  $E_h = +0.416$  volt,  $\mu = 8,700$
- 7  $\text{K}_4\text{Fe}(\text{CN})_6$ ,  $E_h = +0.280$  volt,  $\mu = 8,700$

trode A progressive increase in  $E_h$  from +0.280 to +0.611 volt accompanies an increase from 0 to 100 per cent in  $K_s^-$  relative to  $K_4Fe(CN)_6$ . It thus appears that in  $-Fe(CN)_6$  solutions below  $E_h = +0.42$  volt a  $\mu = 8,700$  is obtained, above  $E_h = +0.46$   $\mu = 11,700$ , and when  $E_h = +0.42 - +0.46$  volt  $\mu = 11,700$  below a critical temperature and 8,700 above that point.

The critical oxidation reduction potential at which the shift in the activation energy of the urease urea system occurs will depend somewhat upon the manner in which the enzyme solution is prepared and also upon the particular compound added to stabilize the potential at a given value. Thus under the conditions of the experiment the sulfite solution was neither strongly reducing nor oxidizing, consequently the temperature characteristic was 11,700 or 8,700, or 11,700 and 8,700 depending upon the enzyme preparation used, the manner in which the solution was prepared, the age of the solution, etc. For the urea phosphate solution  $E_h = +0.357$  volt, this value was not altered significantly by adding an equal volume of urease in such solutions as  $Na_2SO_4$ , KCN, cystine, glycerol, sulfite, and water, but was markedly lowered to +0.28 volt by  $Na_2S_2O_3$  and  $K_4Fe(CN)_6$ . It was appreciably elevated to +0.460 — +0.611 volt by adding urease dissolved in 0.017 M  $H_2O_2$  or 0.068 M  $K_4Fe(CN)_6$ . In general it appears that when the redox potential of the urease urea phosphate system is +0.36 volt or lower the temperature characteristic is 8,700, but when the potential is greater than this  $\mu$  is 11,700. At critical potentials (as in sulfite solutions)  $\mu$  may be either 8,700 or 11,700, or 11,700 below and 8,700 above a critical temperature.

If urease exists in two forms at a given redox potential, then an Arrhenius plot of the data should give a curve that is concave upward. Since this is not the case, urease cannot exist in these two forms simultaneously. At a fixed but critical potential it is assumed that urease is in an unstable condition and can be easily shifted from one form to the other by slight alterations in the thermodynamic equilibria of the system produced by temperature changes or other factors.

#### *Urease Activity Measured by $CO_2$ Evolution As a Function of Temperature*

In the hydrolysis of urea both ammonia and carbon dioxide are liberated. The ammonia is very soluble and is not evolved until the



solution has become very alkaline. The carbon dioxide, some of which is used to neutralize the ammonia, is fairly soluble in phosphate buffer at pH 7. The solution soon becomes saturated, however, and  $\text{CO}_2$  is evolved. This may be measured manometrically, and has been done in carbonate buffer at pH 5.0, by Krebs and Henseleit (1932) who used a Warburg manometer.

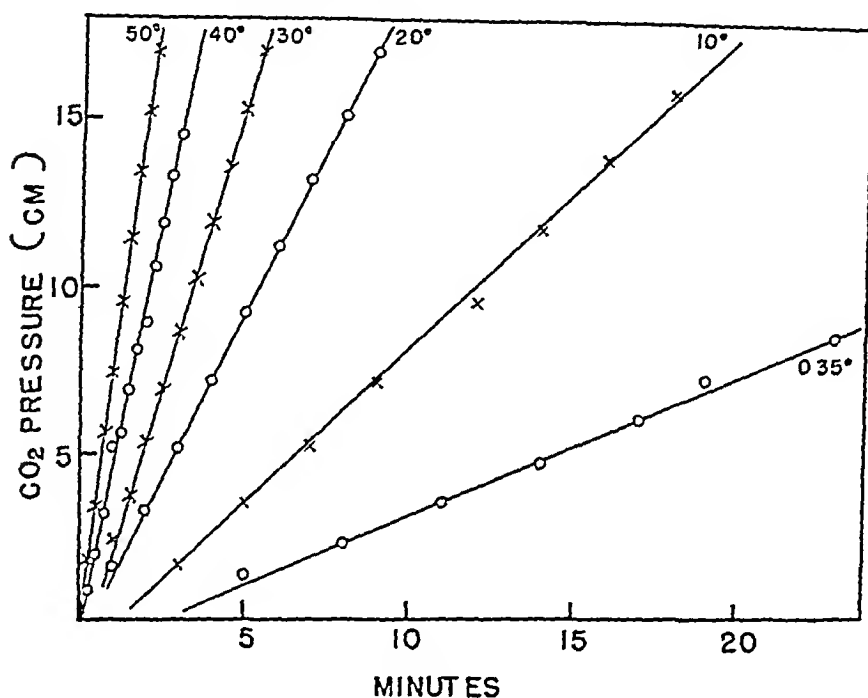


FIG. 6.  $\text{CO}_2$  gas (measured in centimeters on the Barcroft differential manometer) liberated from urea catalyzed by 0.1 per cent Arlco urease in an aerated 0.068 M sulfite solution, plotted as a function of elapsed time in minutes. The pressures have not been corrected for temperature differences. 1 cm = 26 c mm gas.

As was the case in the  $\text{NH}_3$  determination an equal volume of urease solution was added to the urea-phosphate at pH 7.0. 2 ml of each were placed in the flask of the Barcroft differential manometer, and adapted to the temperature of the water bath. Manometer readings were taken at successive intervals after closing the stopcocks. The control flask contained 4 ml distilled water. In all experiments the enzyme solution was 0.1 per cent Arlco urease. A pressure change of

1 mm on the manometer corresponds to a change in volume of 2.6 c mm. Since all the determinations in a given series of temperatures were made within a period of 3-4 hours, no correction was made for

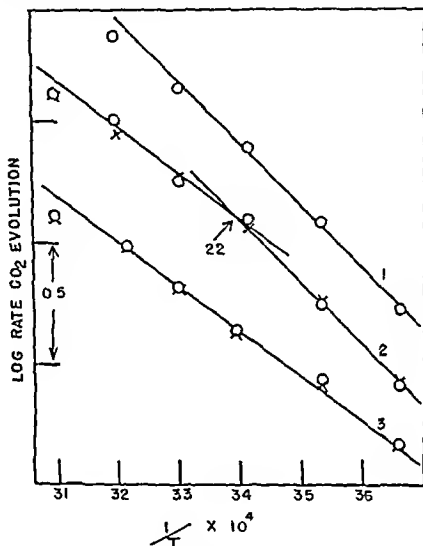


FIG 7 Log rate of CO<sub>2</sub> evolution from urea catalyzed by 0.1 per cent Arlco urease plotted against  $1/T$  \ duplicate experiment carried out in another manometer

- 1 Urease dissolved in 0.068 M sulfite  $\mu = 11,700$
- 2 Same solution after aeration for 2 days  $\mu = 11,700$  below and 8,700 above 22 C
- 3 Urease dissolved in water  $\mu = 8,700$

the slight variations in atmospheric pressure which may have occurred during that time

In Fig 6 are plotted the data on CO<sub>2</sub> evolution as a function of time, in minutes. The enzyme solution was urease dissolved in 0.068 M sulfite aerated for 2 days before using. At all temperatures the

CO<sub>2</sub> liberated is a linear function of time, as was the case for NH<sub>3</sub> production. Rate of evolution was calculated from the slopes of the lines and was multiplied by a factor which corrected for the fact that the gas evolved was measured at different temperatures. When log corrected rate is plotted against  $1/T$  the points fall along two straight lines intersecting at 22°C (Fig 7, curve 2). The slope of the lower line corresponds to a  $\mu = 11,700$ , for the upper  $\mu = 8,700$ . For this same enzyme solution before aeration, the  $\mu$  value is 11,700 over the whole temperature range (Fig 7, curve 1). With 0.1 per cent Arlco urease dissolved in water the data correspond to a  $\mu = 8,700$  over the whole temperature range (Fig 7, curve 3). Similar results were obtained when other substances were added to the enzyme solution, the temperature characteristic was either 8,700 or 11,700, or 11,700 below and 8,700 above a critical temperature. It is clear that the analysis of the data as a function of temperature is the same whether urea hydrolysis is followed colorimetrically measuring NH<sub>3</sub> production, or gasometrically measuring CO<sub>2</sub> evolution.

#### DISCUSSION

The classical work on urease activity as a function of temperature was performed by Van Slyke and Cullen (1914) using soy bean meal, they followed the hydrolysis by distilling off the ammonia and titrating with acid. From their data Euler (1920) calculated a  $\mu = 20,800$  for urease from a  $Q_{10} = 3$  in the temperature range from 30–40°C. This is the value often quoted in text-books. Calculations made from the original data, however, indicate a constant  $\mu = 11,700$  between 10 and 50°C. In a later book Euler (1922) recalculated the same data and obtained a  $Q_{10}$  of 1.91 and a  $\mu$  of roughly 12,000. Observations by Euler and Brandting (1919) on the activity of urease in the temperature range from 30–40°C yield a  $Q_{10}$  of 1.92 corresponding to the same  $\mu$  value. It seems very significant that the  $\mu$  value for soy bean urease is identical with one of the two values associated with jack bean urease.

The phenomenon of a sharp break with one temperature characteristic above and another below a critical temperature when log rate is plotted against  $1/T$  is common when physiological phenomena are involved (*cf* Hoagland, 1936, Sizer, 1936). This sudden change in

temperature characteristic is usually explained by assuming that a change has occurred in the pace maker for the catenary reactions controlling rate (Crozier, 1924). Thus, in a study of sucrose inversion by bakers' yeast, when a break was obtained with two different  $\mu$  values above and below a critical temperature, it was assumed that there exist in yeast two entirely separate enzymes capable of hydrolyzing sucrose, one active below and the other above the critical temperature (Sizer, 1938 *b*). Such breaks have not been encountered heretofore in simple enzyme systems, but have always been associated with vital phenomena. The data on urease cannot be explained by the suggestion that two separate ureases with different  $\mu$  values are present, since crystalline urease has been used which has been recently shown to consist chiefly of a single homogeneous protein with a molecular weight of 483,000 (Sumner *et al.*, 1938). The only explanation of the results which seems likely is that the configuration of the active groups in the urease molecule determines the activation energy. Labile sulfhydryl groups are probably involved, it is suggested that when they are reduced the activation energy is 8,700 calories, but when oxidized (partially or perhaps completely) it is 11,700 calories. This oxidation or reduction accompanied by a change in  $\mu$  is reversible. A similar explanation might apply equally well to a change in temperature characteristic for certain physiological systems. A shift in  $\mu$  value could be accounted for equally well by a shift in pace maker, or a change in the activation energy of a single pace maker caused by an alteration in the configuration of the catalyst for that step in the reaction.

The actual mechanism by which the activation of the urease molecule is modified by the redox potential is not clear. The effective peripheral groups responsible for the urease activity are probably involved in this activation. While the redox potential may determine the configuration of these groups it must not be thought that at a given potential there is an equilibrium set up between those radicals which are in a reduced and those in an oxidized form. Under such conditions with two different forms of urease active independently, an upward concavity should appear in the Arrhenius plot. The degree of curvature would depend upon the redox potential and a break at a critical temperature could not occur. The results could be explained,

however, on the assumption that under a given environmental condition urease exists exclusively in one form or the other. At critical redox potentials the urease may be very labile, and slight changes

TABLE I

The temperature characteristics ( $\mu$  in the Arrhenius equation) are tabulated for urea hydrolysis (as measured by  $\text{NH}_3$  production) catalyzed by a variety of crude and crystalline enzyme preparations dissolved in a number of different solutions

Urease preparation	Enzyme medium	$\mu$ value	Critical temperature	Inactivation temperature
Jack bean meal	$\text{Na}_2\text{SO}_3 + \text{NaHSO}_3$	11,700	—	40°C
Arlico	$\text{Na}_2\text{SO}_3 + \text{NaHSO}_3$	11,700	—	45°
"	Glycerine	8,700	—	50°
"	$\text{H}_2\text{O}$	"	—	>40°
"	$\text{H}_2\text{O}$ , then $\text{SO}_3$	11,700, 8,700	25°C	>40°
Purified "	$\text{Na}_2\text{SO}_3 + \text{NaHSO}_3$	"	26°	65°
Crystalline 1	$\text{Na}_2\text{SO}_3 + \text{NaHSO}_3$	11,700, 8,700	21°	45°
" 2	"	"	"	60°
" 3a	"	"	22°	"
" 3	"	11,700	—	40°
" 3 (partly inactive)	"	8,700	—	40°
" 4a	$\text{H}_2\text{O}$ , then $\text{SO}_3$	11,700	—	>40°
" "	$\text{SO}_3 + 0.034 \text{ M CaCl}_2$	11,700, 8,700	23°	"
" 4a (partly inactive)	"	11,700	—	"
" " " "	$\text{SO}_3 + 0.08 \text{ M CaCl}_2$	"	—	"
" 4a	KCN	8,700	—	"
" 4c	"	"	—	"
" 4c (partly inactive)	"	11,700	—	"
" 4b	$\text{Na}_2\text{S}_2\text{O}_3$	8,700	—	"
" "	Cystine	"	—	50°
" "	$\text{Na}_2\text{SO}_4$	"	—	>40°
" 4a	$\text{H}_2\text{O}$	"	—	"
" 4b	$\text{K}_4\text{Fe}(\text{CN})_6$	"	—	"
" "	$\text{K}_3\text{Fe}(\text{CN})_6$	11,700	—	"
" "	$\text{H}_2\text{O}_2$	11,700	—	"

in the system such as a shift in temperature might cause a conversion of urease from the reduced to the oxidized form or the reverse. With such a mechanism, a shift in the  $\mu$  value might occur at a critical temperature. Dr W J Crozier<sup>1</sup> has suggested the possibility

<sup>1</sup> Personal communication

that in order to be activated the effective peripheral groups of urease must first be oxidized and then reduced, cyclically. With such a system oxidizing or reducing agents would modify one or the other phase of the cycle and in this manner fix the activation energy at 8,700 or 11,700 calories per gram mol.

There are no significant differences between the kinetics as a function of temperature for urease in widely different stages of purity, ranging from a suspension of jack bean meal to crystalline urease which has been once recrystallized (see Table I). In previous experiments on temperature activation of invertase it appears that the medium played no rôle (Sizer, 1937), but this is far from true for crystalline urease, where the addition of certain substances can cause a complete change in the activation energy. The oxidation-reduction potential of the medium may function in fixing the  $\mu$  value of an enzyme system by determining which of the active groups of the enzyme will determine the course of the reaction. Recent work on monomolecular films indicates that urease activity is associated with the hydrophobic surfaces of the urease molecule, the hydrophilic surfaces exerting no measurable activity (Langmuir and Schaefer, 1938). This is consistent with the general point of view that certain groupings in the enzyme molecule are responsible for the enzyme activity. From this present work on the temperature activation of urease it appears that by modifying such active groups not only the enzyme activity is altered but also the activation energy of the enzyme may be changed as well.

#### SUMMARY

1 The hydrolysis of urea catalyzed by jack bean meal has been followed by determining colorimetrically after Nesslerization the ammonia nitrogen, and volumetrically the carbon dioxide liberated at successive intervals during the reaction. During the early part of hydrolysis the rate of ammonia or carbon dioxide liberation is constant for all the urease solutions which were used.

2 When log rate of  $\text{NH}_3$  or  $\text{CO}_2$  formation was plotted against  $1/T$ , the points fell along a straight line, the slope of which corresponded to an activation energy of either 8,700 or 11,700 calories per gram mol. Frequently urease, when dissolved in sulfite solution, was

characterized by an activation energy of 11,700 below and 8,700 above the critical temperature of about 23°C. At high temperatures the plotted points fell off from the curve due to temperature inactivation.

3 Essentially the same results on temperature activation were obtained with crude jack bean meal, Arlco urease, crystalline urease not recrystallized, and crystalline urease once recrystallized. The temperature characteristic which was obtained depended in part upon the composition of the medium. When dissolved in water, or aqueous solutions of glycerine, KCN,  $\text{Na}_2\text{S}_2\text{O}_3$ , cystine,  $\text{Na}_2\text{SO}_4$ , and  $\text{K}_4\text{Fe}(\text{CN})_6$ , the temperature characteristic or  $\mu$  of urease is 8,700. On the other hand, when urease is dissolved in solutions of  $\text{K}_3\text{Fe}(\text{CN})_6$  or  $\text{H}_2\text{O}_2$  the  $\mu$  value is 11,700. When dissolved in a solution containing  $\text{Na}_2\text{SO}_3$  and  $\text{NaHSO}_3$  the  $\mu$  value may be either 8,700 or 11,700 over the whole temperature range, or 11,700 below and 8,700 above 23°C.

4 When crystalline urease is dissolved in varying mixtures of  $\text{K}_4\text{Fe}(\text{CN})_6$  and  $\text{K}_3\text{Fe}(\text{CN})_6$ , the temperature characteristic depends upon the oxidation-reduction potential of the digest. When  $E_h$  is greater than +0.46 volt  $\mu = 11,700$ , when less than +0.42 volt  $\mu = 8,700$ , when between +0.42 - +0.46  $\mu = 11,700$  below and 8,700 above the critical temperature.

5 It is suggested that in reducing or in indifferent solutions the configuration of the urease molecule (as determined especially by SH groups present) is such that the activation energy is 8,700 calories. In oxidizing solutions the urease molecule has been so altered (perhaps by the oxidation of the SH groups) as to be partly inactivated and now has an activation energy of 11,700. Such changes in the urease molecule are reversible (unless oxidation has proceeded too far) and are accompanied by a corresponding change in the activation energy.

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## THE KINETICS OF PENETRATION

### XVIII ENTRANCE OF WATER INTO IMPALED HALICYSTIS

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When a large plant cell, such as *Valonia macrophysa*, Kütz, or *Halicystis Osterhoutii*, Blinks and Blinks, is impaled on a capillary the restriction on the uptake of substances from the external solution, due to the limited elasticity of the cellulose wall, is removed, and as a result water and electrolyte enter at a much greater rate than into the intact cell. In the case of *Valonia*,<sup>1</sup> for example, the rate of uptake increases about 15 fold. The technique of determining the increase in volume, which is practically equivalent to the increase in the quantity of water in the cell, consists in measuring the rise of liquid in a capillary. This is so simple that it seemed worth while to measure the rate of entrance of water from diluted sea water into individual *Halicystis* cells to determine the absolute rate of water migration in *Halicystis*, and if possible to gain information about the structure of the protoplasm.

The experiments were carried out in Bermuda in the winter of 1936-37 at the Bermuda Biological Station.

#### EXPERIMENTAL

The method of impaling *Halicystis* cells has been described by Blinks,<sup>2</sup> and the assembly used when the rise of liquid in the capillary is to be measured has already been discussed by us.<sup>1</sup> In the present case the experiments were carried out at 17°C  $\pm$  1°C which was the temperature of the sea water flowing through the tray in which the bottles containing the cells were immersed. Before impalement the volume of each cell was determined individually by the method pre-

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<sup>1</sup> Jacques A G, *J Gen Physiol*, 1938-39, 22, 147

<sup>2</sup> Blinks, L R, *J Gen Physiol* 1929-30, 13, 223

viously described<sup>3</sup> After impalement each cell was allowed to stand in normal sea water at least 48 hours, during which time the rate of increase of the volume of the sap was measured, to make sure that it followed an approximately linear course, since from other experiments, to be described in another paper, this appears to be the regular behavior in normal sea water

The sea waters to which the cells were exposed ranged from 90 per cent down to 30 per cent and the length of exposure was such that the amount of water entering would not dilute the sap by as much as 10 per cent usually the calculated dilution was not more than 5 per cent<sup>4</sup> Each cell was exposed to all the diluted sea waters and between exposures each was allowed to stand at least 24 hours in normal sea water in order to recover from the dilution of the sap During this time the sap volume decreased somewhat, as shown by the fall of liquid in the capillary But within a comparatively short time the entrance of electrolytes and water was resumed and when it attained a linear rate about the same as that observed before the cell was exposed to dilute sea water, recovery was considered to be complete

### RESULTS

The results of a typical experiment are given in Fig 1 This shows the rate of increase in volume of the sap of a single cell on exposure to dilute sea waters The increase in volume has been calculated from the formula

$$\Delta V = H\pi r^2$$

where  $H$  is the increase in the height of liquid in the capillary which was measured by means of a micrometer caliper reading to 0.02 mm, and  $r$  is the radius of the capillary obtained by direct microscopic measurement of the diameter of the capillary, by means of a micrometer ocular

Without any assumptions as to the nature of the protoplasm, we may assume that

$$\frac{dQ}{dt} = \frac{k_1 A}{h} (N_o^{H_2O} - N_i^{H_2O}) \quad (1)$$

<sup>3</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, 15, 537

<sup>4</sup> The initial cell volume and the increase in volume being known it was possible to calculate the dilution if no electrolyte were lost by the cell. Probably, however, a little electrolyte was lost but the amount was not large even when the sea water was diluted to 30 per cent. Some data are given on this point farther on in the paper

where  $N^{\text{H}_2\text{O}}$  is the concentration of water on the mole fraction basis,  $A$  is the surface area, and  $h$  the thickness of the membrane, and  $o$  and  $i$  refer to the sea water and sap respectively

If we assume that there are non aqueous surface layers at the sap protoplasm and sea water protoplasm interfaces we must take into account the process of solution of water in the non aqueous layers

From Osterhout's treatment of the guaiacol model we assume that at each interface there are in contact unstirred layers, one in water and the other in the protoplasm, and that at the immediate interface there are thin regions in each layer where all the molecular species in both phases are in equilibrium across the boundary For any species, say  $r$ , we can write for the sea water protoplasm interface

$$S'_{eop} N'_{eop} = N'_{epo}$$

and for the sap-protoplasm interface

$$S'_{sip} N'_{sip} = N_{spi}$$

where  $eop$  and  $epo$  refer to the portions of the unstirred layers, in sea water and protoplasm respectively, where the equilibrium prevails, and  $S'$  is the partition coefficient of  $r$  If we are dealing with water which is present in much greater abundance than any other species we may assume that there is no gradient of water across the unstirred layers so that

$$N^{\text{H}_2\text{O}}_{eop} = N^{\text{H}_2\text{O}}_o \quad \text{and} \quad N^{\text{H}_2\text{O}}_{sip} = N^{\text{H}_2\text{O}}_i$$

Hence the rate of entrance of water is equal to the flux of water across the non aqueous protoplasmic layers, and if we can treat these as a single thin layer, then the flux is given by

$$\frac{dQ}{dt} = \frac{k_1 A}{h} (N^{\text{H}_2\text{O}}_{epo} - N^{\text{H}_2\text{O}}_{spi}) \quad (2)$$

or

$$\frac{dQ}{dt} = \frac{k_1 A}{h} (S'_{eop} N^{\text{H}_2\text{O}}_o - S'_{sip} N^{\text{H}_2\text{O}}_i) \quad (3)$$

If the water added to the sap mixes without expansion or contraction of volume we can write for the sap  $\frac{dV}{dt}$  for  $\frac{dQ}{dt}$

Assuming that the solutions are ideal dilute ones, we may now apply the gas laws, whence

$$P_e V_e = P_t V_t = P_s V_s = \text{a constant} \quad (4)$$

where  $P$  is the osmotic pressure,  $V$  is the volume of the sap,  $e$  refers to conditions at the theoretical<sup>5</sup> end of the experiment when sap and sea water are in osmotic equilibrium,  $t$  to any time  $t$  during the exposure, and  $s$  to the beginning of the exposure (zero time)

But

$$N^{\text{H}_2\text{O}} = 1 - \Sigma N^{\text{sol}}$$

where the index "sol" means solute

Now for 1000 ml of a dilute solution of an electrolyte, we may say that

$$P = \frac{\iota m RT}{V} = \frac{\iota n^{\text{sol}} RT}{1000}$$

where  $m$  is the molar concentration of the solute,  $V$  is the volume of the solution, assumed to be equal to the volume of the solvent (water) alone,  $\iota$  is the van't Hoff coefficient, and  $n^{\text{sol}}$  is the number of moles of solute Or

$$P = \frac{\iota}{V_m} \frac{n^{\text{sol}} RT}{n^{\text{H}_2\text{O}}}$$

where  $V_m$  is the molal volume of the solvent =  $1000 - 55.5 \simeq 18.0$ , and  $n^{\text{H}_2\text{O}}$  is the number of moles of water

But in an aqueous solution in which  $n^{\text{H}_2\text{O}}$  is large compared with  $n^{\text{sol}}$  we can write

$$\Sigma N^{\text{sol}} = \frac{\Sigma n^{\text{sol}}}{n^{\text{H}_2\text{O}}}$$

whence

$$P = \frac{\iota RT \Sigma N^{\text{sol}}}{V_m}$$

---

<sup>5</sup> Theoretical in the sense that the experiments were never allowed to go to equilibrium and it is probable that if they had the measured  $V_s$  would have been smaller than the theoretical  $V_s$  for the reason that as the exposure progressed some electrolyte would have been lost by the cell

From this point on  $\frac{iRT}{V_m} = \omega$  another constant<sup>6</sup>

Then

$$\frac{dV}{dt} = \frac{k_1 A}{h} \left\{ S_{exp} \left( 1 - \frac{P_s}{\omega} \right) - S_{exp} \left( 1 - \frac{P_t}{\omega} \right) \right\} \quad (5)$$

dividing through by  $S_{exp}$  and rearranging we get

$$\frac{dV}{dt} = \frac{k_1 A S_{exp}}{h} \left\{ b - 1 + \frac{P_t}{\omega} - \frac{P_s}{\omega} \right\} \quad (6)$$

where  $b$  equals the ratio  $S_{exp} - S_{exp}$  Or substituting for  $P$

$$\frac{dV}{dt} = \frac{k_1 A S_{exp}}{h} \left\{ b - 1 + \frac{\eta V_s - \eta b V_t}{V_s V_t} \right\} \quad (7)$$

where  $\eta = P_s V - \omega$  Putting  $b = 1$  and integrating gives

$$\frac{k_1 A S_{exp}}{h} = \frac{V_s \omega}{P_s V_s} \left[ V_s - V_t + 2.3 V_s \log \frac{V_s - V_s}{V_s - V_t} \right] \quad (8)$$

Putting  $k_1 S_{exp} - h = k$  the equation becomes

$$\frac{k}{\omega} = \frac{V}{i P_s V A} \left[ V_s - V_t + 2.3 V_s \log \frac{V_s - V_s}{V_s - V_t} \right] \quad (9)$$

which is practically identical with that of Northrop<sup>7</sup> It differs only in containing  $S_{exp}$  and in that the term  $\omega$  is explicit  $\omega$  is the proportionality constant relating osmotic pressure to the concentration of the solution expressed as mole fraction of solute Northrop's equation was obtained by integrating

$$\frac{dV}{dt} = \frac{k A P V_s}{h} \left( \frac{1}{V_t} - \frac{1}{V_s} \right) \quad (10)$$

Equation (9) has been used in the present work to calculate permeability constants of water entrance In these calculations  $V_s$  was

$$^6 \quad \omega = \frac{iRT}{V_m} = \frac{1.8 \times 1985 \times 4.015 \times 10^{-3} \times 290.6}{0.018} = 2320 \text{ atmospheres}$$

<sup>7</sup> is taken as 1.8 which is the approximate value for the 0.61 M sodium chloride solution, to which *Halocystis* sap very nearly corresponds

<sup>7</sup> Northrop, J. H., *J. Gen. Physiol.* 1927-28 11, 43

obtained directly by a displacement measurement of water,  $V_i = V_e + \Delta V$ .  $\Delta V$  was measured<sup>3</sup> directly from the rise in the capillary.  $A$  was calculated from  $V_e$  on the assumption that the cell is spherical and since care was taken to use cells as nearly spherical as possible this assumption is a reasonably good one.  $P_e$  was calculated from the average freezing point lowering of *Halicystis* sap (which is 2.059°C) for the relationship

$$P = \frac{L\rho\Delta T}{T}$$

where  $L$  is the latent heat of fusion of the solvent,  $\rho$  is the density of the solution,<sup>8</sup>  $\Delta T$  is the lowering of the freezing point, and  $T$  is the freezing point of the solution. Taking  $L = 80$  cal and 1 gm cal = 41.3 cm<sup>3</sup> atmospheres,

$$P_e = \frac{80 \times 1.026 \times 2.059}{271.04} = 0.6147 \text{ gm cal/cm}^3 = 25.39 \text{ atmospheres}$$

$V_e$  has been calculated from  $V_i$  on the assumption that  $P_e = P_o$ , where  $P_o$  is the osmotic pressure of the sea water to which the cell is exposed. Then

$$V_e = \frac{V_i P_e}{P_o}$$

Assuming that the van't Hoff coefficient remains constant over the range of dilute sea waters employed,

$$V_e = \frac{V_i C_e}{C_o}$$

In this calculation  $C_e = 100$  per cent and  $C_o =$  per cent composition of the dilute sea water.

Values for the constant  $k/\omega$  are given in Table I. These are calculated from the data of Fig. 1. Neglecting the first and second minutes, which show large trendless irregularities, it will be observed that the "constant" remains reasonably constant in each sea water dilution for

<sup>8</sup> We have assumed that *Halicystis* sap is a 0.6 M solution of sodium chloride, the density of which at 0°C is according to interpolation from Baxter and Wallace's data 1.026. This value of  $\rho$  has been used in our calculation.

at least the first 10 minutes of the exposure. Thereafter it decreases fairly regularly in most cases, and the decline is too great to be merely the result of experimental error. To save space, values after the first 10 minutes have been omitted but the value from the last reading and the time are given.

TABLE I

Constants\* ( $k/\omega \times 10^5$ )

$$k/\omega = \frac{V}{V_0 P A t} \left( V - V_1 + V \cdot 2.3 \log \frac{V - V_1}{V - V_2} \right)$$

$$V = 0.423 \text{ cm.}^3 \quad P = 25.39 \text{ atm} \quad A = 2.724 \text{ cm.}^2$$

Time	Per cent concentration of sea water						
	30	40	50	67	75	83	90
min							
1	1.42	2.91	2.18	2.27	3.53	2.52	3.31
2	2.91	2.52	3.01	2.89	3.58	3.42	2.91
3	2.00	2.49	2.64	3.43	3.18	3.42	2.72
4	2.01	2.37	2.95	3.02	2.87	3.20	—
5	2.17	2.36	2.97	3.21	2.74	2.91	3.32
6	2.13	2.33	2.84	3.00	2.76	3.31	3.15
7	2.01	2.30	2.81	2.94	2.76	3.20	2.94
8	2.00	2.39	2.77	2.96	2.78	3.41	3.14
9	1.92	2.19	2.71	3.01	2.76	3.22	3.12
10	2.03	2.31	2.66	3.09	2.74	3.21	3.05
	1.88	2.03	2.32	2.53	2.28	2.85	2.72
	†(20)	(20)	(35)	(60)	(75)	(90)	(90)
Average $k/\omega$ between 3 to 10 minutes	2.04	2.34	2.79	3.08	2.82	3.24	3.06

\*  $k = k_1 S_{wp} / h$ , where  $k_1$  is the true permeability constant;  $\epsilon$  the number of cubic centimeters of water entering the cell per minute, through 1 cm<sup>2</sup> of a surface layer 1 mm thick under a difference of osmotic pressure of 1 atm.

† Figures in parentheses are times in minutes at which last readings were made.

Neglecting the last part of each run and omitting the first 2 minutes, which show the large deviations, not unusual at the start of kinetic processes, we have calculated the average value of  $k/\omega$  (see page 747) for the period between 2 and 10 minutes. The results are given in Table I and have been plotted in Fig. 2 which contains, in addition,



the values of  $k/\omega$  against per cent concentration of sea water for other cells for which sufficient data are available, and an average curve for all points common to these cells. Although there are rather large deviations, each curve and the average curve show unmistakably an abrupt drop as the concentration of sea water drops below 50 per cent. This is clearly brought out in Fig 1, where the curves for 40 and 30 per cent sea water coincide with each other and approximately with the 50 per cent curve. Above 50 per cent  $k/\omega$  is either constant or perhaps it drops very slowly.

We must now consider whether the equation here used for the calculation of  $k/\omega$ , which indicates that it suffers some change with time at constant external sea water concentration, and also some change with sea water concentration, really represents accurately the behavior of water.

In his discussion of the diffusion of water into *Arbacia* eggs, Northrop pointed out that it might be necessary to take into account (a) the increase in the surface and decrease in the thickness of the membrane, or (b) if a pore system is in question the increase in the diameter of the pores and the decrease in their length, as the cell increases in volume.

For both cases the same equation was derived and integrated, and a somewhat similar equation was obtained by Lucké, Hartline, and McCutcheon,<sup>9</sup> also for the swelling and shrinkage of *Arbacia* eggs. Both their equation and Northrop's new equation, gave better constants than the original Northrop equation. Furthermore,  $k/\omega$  calculated on the basis of Northrop's original equation decreased with the decrease in concentration of the sea water. This effect disappeared when the new equation, taking into account the changes in the dimensions of the membrane, was employed. Because in our work the value of  $k/\omega$  calculated from Northrop's original equation decreased rather abruptly when the sea water concentration was 50 per cent or less, it seemed fair to inquire whether we should attempt to apply Northrop's new equation to our data. On reflection this seemed to be an unjustified step, since in the case of an impaled cell there is

<sup>9</sup> Lucké, B, Hartline, H K, and McCutcheon, M, *J Gen Physiol*, 1930-31, 14, 405

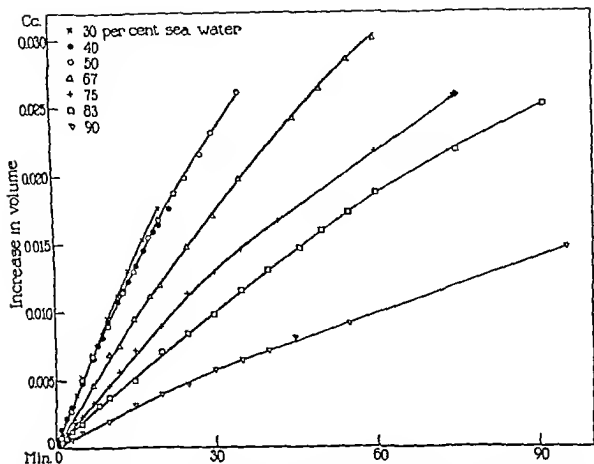


FIG 1 Rate of entrance of water into a single cell of *Halicystis Osterhoutii* from dilute sea water. The curves are drawn free hand to give an approximate fit.

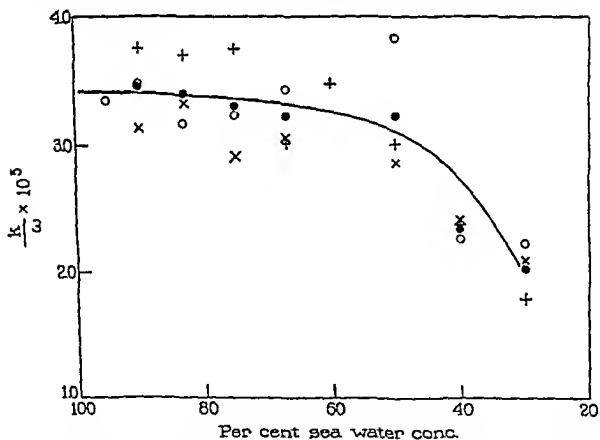


FIG 2 Velocity constants plotted against per cent concentration of sea water. The curve is drawn free hand to give an approximate fit.

no evidence that the surface area changes at all <sup>10</sup> Indeed this is an outstanding advantage of this type of experiment The equation was therefore not used and we are satisfied with the conclusion on the basis of the old equation that the absolute rate does decrease abruptly somewhere around 50 per cent sea water concentration

If it were possible to apply corrections for the deviations of the gas laws, which apply strictly to ideal dilute solutions only, some rectification of  $k/\omega$  as a function of time might be possible Unfortunately not much progress which is thermodynamically sound can be made in this direction But it is possible to show that probably any rectification which would be achieved by correcting for deviation from ideal will be negligible

Strictly the relationship of equation (4) should be

$$\frac{P_z V_z}{\phi_z} = \frac{P_t V_t}{\phi_t} = \frac{P_e V_e}{\phi_e} = \text{constant} \quad (4a)$$

where  $\phi$  is the osmotic coefficient of Bjerrum <sup>11</sup> This leads to

$$\frac{dV}{dt} = \frac{kAP_z V_z}{h\phi_z} \left( \frac{\phi_t}{V_t} - \frac{\phi_e}{V_e} \right) \quad (4b)$$

for equation (10)

This equation cannot be integrated readily because  $\phi$  in concentrated solution is not simply related to the concentration or the osmotic pressure of the solution <sup>12</sup>

<sup>10</sup> It is possible that in the course of an exposure of a cell to dilute sea water, the rise of the liquid in the capillary might cause actual stretching of the membrane But since this rise was never more than 2 cm this effect may be neglected

<sup>11</sup>  $\phi$  is defined by  $\frac{P_{\text{ideal}} - P_{\text{real}}}{P_{\text{ideal}}} = 1 - \phi$  It is related to the  $j$  function used by Lewis and Randall (Lewis, G N, and Randall, M, Thermodynamics, New York, McGraw-Hill Book Company, Inc, 1923, 286)  $1 - \phi = j$

<sup>12</sup> In dilute solutions of uni-univalent electrolytes, according to Lewis and Linhart (Lewis, G N, and Linhart, G A, *J Am Chem Soc*, 1919, 41, 1951)  $j = \beta m^{\frac{1}{2}}$ , where  $m$  is concentration in molal terms and  $\beta$  is a constant Unfortunately as  $m$  increases even in pure solution of uni-univalent electrolytes, the relationship between  $j$  and  $m$  becomes very complex Scatchard (Scatchard, G, *Chem Rev*, 1936, 19, 309) following Debye has derived quite different equations relating  $\phi$  and the ionic strength of concentrated solutions either pure or mixed, but his equations cannot be applied to the present case, since they introduce new variables

About the best we can do is to show that probably the osmotic coefficients over the range of sea water and sap concentrations used are so little different from each other that the correction can be neglected

If we assume that the sap of *Halocystis* is approximately 0.6 M NaCl solution<sup>11</sup> the osmotic coefficient, according to Scatchard and Prentiss<sup>11</sup> freezing point measurements, is 0.91. And if in the course of its exposure it is diluted to the theoretical limit, so that  $P \approx P$ , then in 30 per cent sea water its concentration should drop to about 0.18 M at which the osmotic coefficient is approximately 0.92. This is the maximum sweep which would occur in the experimental work. Accordingly we are justified in assuming that  $\phi$  is a constant in the present case, and as such it cancels out in equation (4 b)

The decrease of  $k/\omega$  with time might be due to the slow loss of electrolyte by the cell, since the protoplasmic membrane is clearly not a true semi permeable membrane. The movement of electrolyte may be so slow in comparison with the movement of water that at the start (within the limits of experimental error) the cell appears to act as a true osmometer and a reasonably accurate value for the permeability constant is obtained. Only after a comparatively long time does the effect of the loss of electrolyte appear. In the present experiments a few tests of the loss of electrolyte have been made by exposing cells to dilute sea water and then comparing the calculated concentration of halide with the actual concentration found from analysis.

In making these calculations the concentration of halide of impaled cells 24 hours after exposure to normal sea water was taken as 0.5975 M. This is the average of 4 cells. But the individual measurements varied from 0.6020 M to 0.5900 M. Because of this variation the calculated values are somewhat uncertain, but in every case they

<sup>11</sup> The composition of *Halocystis* sap according to Blinks and Jacques (Blinks, L. R., and Jacques A. G., *J. Gen. Physiol.*, 1929-30, 13, 733) is

Chloride	0.6028 moles per liter
Sodium	0.5570 " "
Potassium	0.0064 " "
Calcium	0.0080 " " "
Magnesium	0.0167 " " "
Sulfate	Trace
Total cations	{ 0.5881 " " "
	{ 0.603 gm equivalents per liter

<sup>11</sup> Scatchard G., and Prentiss, S. S. *J. Am. Chem. Soc.* 1933, 55, 4355

were higher than the values actually determined, which suggests that some electrolyte had been lost. For example, a cell in 30 per cent sea water for 30 minutes increased its sap volume in such a way that  $\frac{V_o}{V_o + \Delta V}$  of 0.5975 M (the calculated value) was equal to 0.5735 M, but the actual value was 0.5520 M. The calculated value may be a little high for the reason that  $V_o$ , the original cell volume, was that of the intact cell but since the cell contracts on impalement, by spurting, a smaller  $V_o$  should be used in the calculation. It is estimated that the cell volume may shrink about 20 per cent in this way, at the start, and if we apply a 20 per cent correction<sup>15</sup> to  $V_o$ , the "corrected" calculated value of the halide concentration becomes 0.5681 M. The figures therefore indicate that there is some loss of electrolyte and that it is small. But it may possibly account for the decrease in  $k/\omega$  with time. But, on the other hand, the large drop in the "constant" when the external sea water concentration is below 50 per cent clearly cannot be accounted for in this way, since the rate of loss of electrolyte is too slow.

If the water passes through the protoplasm by diffusing in pores we should expect the rate of increase of volume  $dV/dt$  to increase steadily as the concentration gradient of the water between sea water and sap increases. That is  $k/\omega$  should remain constant. But, of course, if for any reason, as the sea water is made more dilute,  $h$  increases or the radius of the pores decreases,<sup>16</sup> then  $k/\omega$  should decrease. But the only probable way in which either  $h$  or the pore radius can change is by an increase in the volume of the non-aqueous part of the protoplasm due to the taking up of more water as the water concentration of the sea water increases.

But as water is soluble in the non-aqueous protoplasm we can

<sup>15</sup> It may be suggested that this decrease in volume should be taken into account in estimating  $k/\omega$ . This is strictly true but since  $V_o$  is estimated from  $V_s$  the discrepancy due to neglecting this loss is partially wiped out. For example, if we take the case of the cell in 30 per cent sea water. Instead of taking  $V_o = 0.423$  cc we take it as 0.3384, i.e., 20 per cent less. The  $k/\omega$  at 3, 5, and 10 minutes are respectively 0.00238, 0.00255, and 0.00237. Thus while we get slightly different values for the "constant" these also show no trend.

<sup>16</sup> If diffusion is in pores  $A$  is equivalent to the area of the pores, rather than the total area of the membrane.

readily conceive that it is transferred between the sap and sea water without passing through pores. The decrease in the value of the constant might then be accounted for on the assumption that the partition coefficients change.

#### SUMMARY

The rate of entrance of water into impaled cells of *Halocystis Osterhoutii*, Blinks and Blinks, has been determined directly by measurements of the rise of sap in a capillary for dilute sea waters (containing between 90 and 30 per cent sea water).

The velocity constant remains reasonably constant down to 50 per cent sea water but it decreases markedly in lower concentrations.



## THE KINETICS OF PENETRATION

### XIX ENTRANCE OF ELECTROLYTES AND OF WATER INTO IMPAIRED HALICYSTIS

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In a previous paper<sup>1</sup> it was shown that when cells of *Valonia macrophysa*, Kutz, are impaled on a capillary and immersed in normal sea water the rate of entrance of water and of electrolyte is about 15 times as great as with intact cells. In the present paper it is shown that similar results are obtained with impaled *Halicystis Osterhoutii* (Blinks and Blinks)

The experiments were carried out in Bermuda at the Bermuda Biological Station in the winter of 1936-37

#### EXPERIMENTAL

The setup for the measurement of the increase in volume for single impaled cells has already been described in a previous paper,<sup>1</sup> and the same technique was followed here. Briefly it consists in impaling a cell on a very thin walled capillary drawn on the end of a tube of capillary bore, exposing it to normal sea water and determining the rate at which the volume increases from the increase in height of the sap in the capillary tube. The rise in height was determined by means of a micrometer caliper reading to 0.02 mm and the volume was calculated from the formula

$$V = \pi r^2 h$$

where  $r$  is the radius of the capillary and  $h$  is the capillary rise. The radius for each capillary tube was determined by direct measurement of the diameter with a microscope fitted with an ocular micrometer. The temperature of the sea water during the exposure was maintained at  $17^\circ\text{C} \pm 1^\circ\text{C}$  by immersing the bottles

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<sup>1</sup> Jacques, A G, *J Gen Physiol*, 1938-39 22, 147



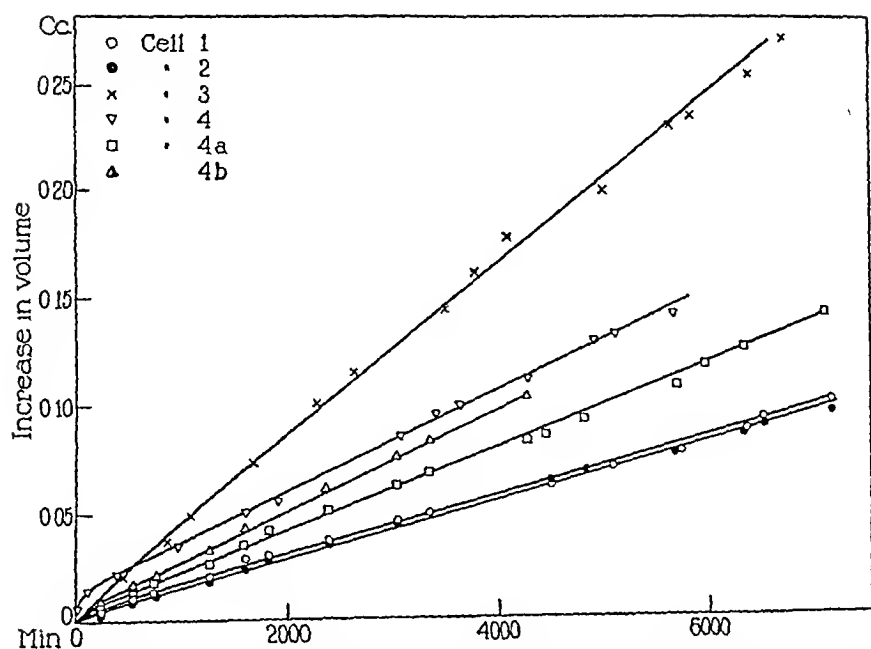


FIG 1 Rate of increase of sap volume of impaled *Halocystis* in normal light (i.e. natural succession of daylight and darkness) The curve for Cell 4 ( $\nabla$ ) starts immediately after impalement, the other curves start 24 hours or more after impalement

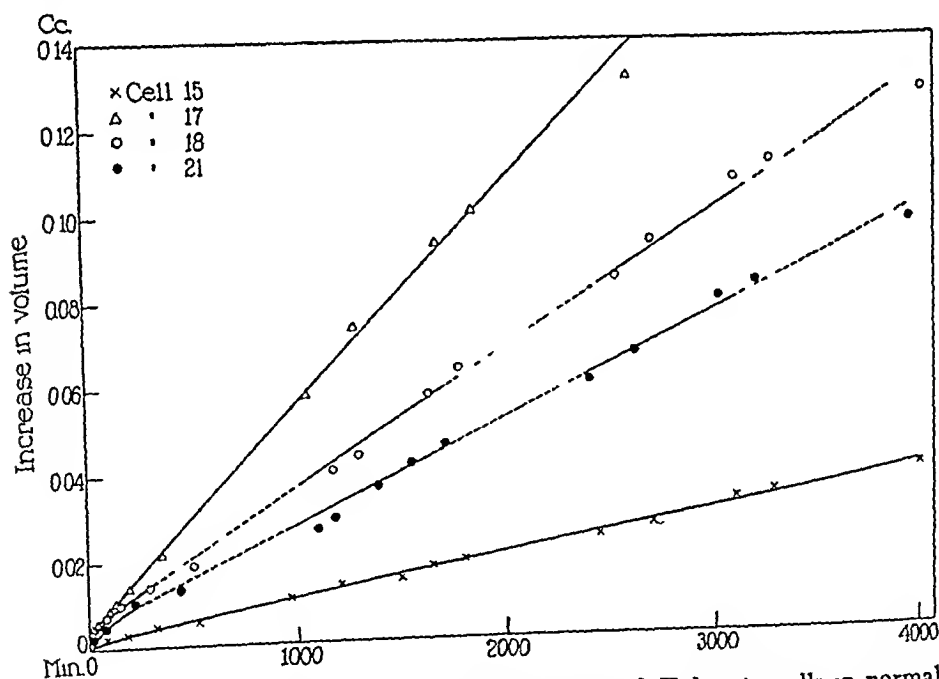


FIG 2 Rate of increase of sap volume of impaled *Halocystis* cells in normal light (natural succession of daylight and darkness) from the moment of impalement Note the initial rapid non-linear increase at the start The curves for Cells 18 and 21 are drawn so as to show the falling off in rate during the hours of darkness and the gain in daylight The broken portions represent 12 hours of darkness, the solid portions 12 hours of light In drawing the curve these differences in rate have been disregarded so that the curve is approximately linear

containing the cells in a large shallow tray through which passed a rapid current of sea water from the salt water pumping system of the Biological Station

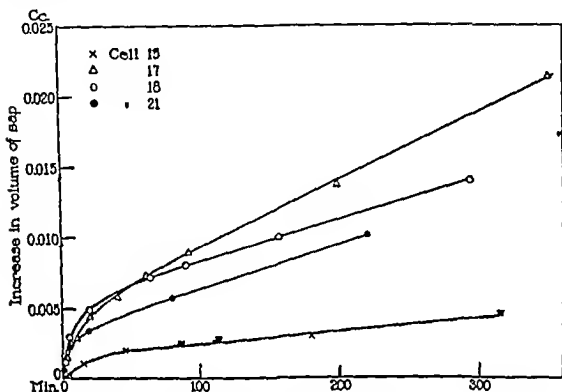


FIG 3 A large scale representation of the early part of the curves of Fig 2, illustrating the initial non linear rate of volume increase

TABLE I

Increase in Volume of Sap and in Moles of Halide in Impaled Cells of *Halicystis*

Cell No	Days	Increase in volume of sap	Increase in volume of sap	Area of surface	Increase in moles halide per day	Increase in volume of sap per day
		cc	per cent	cm <sup>2</sup>	per cent	per cent
1	5 0	0 095	33 0	2 108	6 6	6 6
2	5 0	0 101	31 0	2 328	6 2	6 2
3	4 6	0 269	45 7	3 394	9 4	9 9
4	3 9	0 141	17 5	4 189	4 6	4 5
Average						6 8

The increase in the moles of electrolyte in the sap (equal to molar concentration times volume increase in liters) was determined as the increase in the moles of halide. Since the cell sap of *Halicystis Osterhoutii* is chiefly a solution of sodium chloride it was considered unnecessary to determine sodium and potassium as well in order to calculate moles of electrolyte.

The halide analyses were carried out electrometrically by titration with standard silver nitrate. The volume of each cell to be impaled was first determined by the method previously described and the number of moles of electrolyte originally present was calculated by using for the halide concentration in every case, the average halide concentration of a number of unimpaled cells from the same collection as the impaled cells. Since the natural variation in halide concentration is comparatively small this method gives sufficiently accurate results.

For the determination of the rate of increase in volume and in moles a group of unimpaled cells was kept under the same conditions as the impaled cells. The volume of these unimpaled control cells was determined in the manner previously described.<sup>2</sup>

A difficulty which arose constantly in these experiments was the decrease in the volume of sap within the cell vacuole at the moment of impaling. Blanks has already spoken of the difficulties associated with impalement of *Halocystis*, due to the tendency of the envelope to tear rather extensively. This means that in many cases some sap escapes round the capillary as the cell is pierced, hence it is hard to estimate the shrinkage of the vacuole. In a few cases cells were impaled without the loss except into the capillary tube and from measurements of this initial capillary rise in these cases it was possible to determine the shrinkage of the vacuole. It is variable, but appears to be between 15 and 25 per cent of the unimpaled volume.

In calculating the per cent increase in volume and in moles this has been taken into account by making a correction of the initial volume of 20 per cent. It is interesting that the difficulty just discussed is almost entirely absent in *Valonia*. In the first place, on impalement the vacuole shrinks only a very little, and, in the second, in nearly all cases of successful impalement the cell wall clings to the capillary so closely that there is no spurting.

In most cases spurting occurred with *Halocystis* cells, and before measurements of the increase in volume were started the cells were allowed to recover in normal sea water for 24 hours or more. The volume time curves for a number of these cells are given in Fig. 1. The curves are typical and the cases chosen were selected so as to avoid too many overlapping points.

In a number of cases we were successful in impaling cells without loss around the capillary. In some of these cases measurements were started at once, in order to determine the shape of the curves at the start. The results for some of these experiments are given in Fig. 2 and on a larger scale in Fig. 3 for the early part of the runs. One of these cells, No. 4, has been included in Fig. 1 for comparison.

Table I gives the data for the beginning and end of four experiments for which sap analyses are available. In 17 other cases not reported

<sup>2</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, 15, 537

in detail the cells were transferred to other experiments and no sap analysis is available

The control cells (unimpaled) gained 6.71 per cent in volume of sap in 8 days and 17.76 per cent in 18 days. The average daily increase was taken as 0.99 per cent.

#### DISCUSSION OF RESULTS

Figs. 1, 2, and 3 show that generally the behavior of the *Halicystis* cells was like that of the *Valonia* cells. Immediately after impalement there was a rapid increase in volume which was succeeded after a few hours by a slower increase, approximately linear with time, which lasted until the end of the experiment. The longest experiments described in this paper lasted 5 days, after which the cells were used in other experiments, and it seems not improbable that in the case of *Halicystis*, barring accidents, the linear increase in volume might go on indefinitely.<sup>3</sup> In the case of *Valonia* the experiments were all terminated by the end of 48 hours because of the difficulty of keeping the capillary from plugging for long periods. But it seems probable that with *Valonia* also, as long as the capillary was kept free, the linear rate of increase of volume would continue.

In the case of *Halicystis*, although the general course of the curve after the first rapid uptake of water is linear, the curves are actually a series of waves. This is clearly brought out in Fig. 2 where a rather large scale is used. Some of the deviations from the linear curve are undoubtedly the result of experimental error but there is a certain regularity about them which we are able to identify definitely with the illumination. During the dark hours, the rate dropped off appreciably but it recovered promptly in daylight so that the net result was nil and the average curve is a straight line.

In order to bring out the periodic nature of the process under the influence of light two of the curves have been roughly divided into light and dark periods, the dotted portions representing 12 hours of darkness, roughly from 7 P. M. to 7 A. M., and the solid portions each

<sup>3</sup> As a matter of fact cells used in other experiments lasted up to 20 days on the capillary and it is our impression that but for the unavoidable disturbances incident to the measurement of the capillary rise they might have lasted much longer.

representing 12 hours of light. The decrease in darkness and the increased rate in the light is fairly evident. These findings are in agreement with other light and dark experiments to be described in another paper.

Referring to Table I we find that the rate of increase of volume and of moles of halide (equivalent to moles of electrolyte) were parallel. This means that the halide concentration remained practically constant. The average percentage increase in volume per day in this group was 6.8 per cent, or if we apply a correction of 20 per cent to the initial volume of the cells to account for the shrinkage on impalement, the daily percentage increase in volume was 8.5 per cent.

This may be compared with the average daily increase of volume of 9.08 for 14 cells all drawn from an old collection, or on the corrected basis of 11.35 per cent. We have elected to compare these 14 cells which include the 4 of Table I because these were of the same collection as the unimpaled cells. The remaining 7 cells were of a much more recent collection and this apparently has some effect on the rate, since the corrected daily volume increase in this case was 19.9 per cent or rather less than twice as much as with the older cells. This difference may have some significance.

Neglecting these last younger cells for the reason that we have no figures for the rate in young unimpaled cells we see that volume increase and hence electrolyte increase was between 9 and 11 times as fast in the impaled cells as in the intact cells. This may be compared with the 15-fold increase observed when *Valonia* cells were impaled. We may say that the increase in rate produced by impalement is approximately the same in *Valonia* and in *Halocystis*.

In the case of *Valonia* we were not able to compare rates with the surface area, because the cells vary so much and are frequently so irregular in shape that we cannot relate the surface to the volume. In the case of *Halocystis* we can assume that the cells are spherical, particularly if they are small cells or have been kept a long time in the laboratory, under which condition they tend to round out to spheres. And if they are spheres we can calculate  $A$ , the surface area, from the volume.

From the calculations it appears that there is very little correlation between the surface area and the rate of uptake of water and electro-

lytes. However, when different cells are compared the average rate of increase of volume per day per square centimeter of surface for the first 14 cells was 0.013 cc and for the other 7 cells 0.022 cc, or nearly twice as great. In the latter case, as pointed out already, this may be correlated with the time the cells remained in the laboratory.

In the case of the unimpaled cells the total surface area for 4 cells from the original volumes was 9.658 cm<sup>2</sup>. And ignoring the small increase in surface area during the volume increase this means that the average volume increase per day per square centimeter of surface was 0.015 cc, so that on this basis the rate is increased somewhat less than 9 fold due to impalement. In the calculation of the surface no correction was made for volume shrinkage on impalement. Such an allowance would raise the figure somewhat, possibly to nearly 10.5-fold, but we need not, in view of other uncertainties, make the calculation here. On either volume or surface basis therefore we can conclude that the rate of uptake of electrolyte and of water increases about 10 fold when the restriction due to the cellulose wall is removed by impalement.

As in the case of *Valonia* the osmotic concentration<sup>4</sup> of the sap in the intact cell is slightly greater than that of the sea water. The freezing point of the sea water passing through the Biological Station pumping system varies slightly from time to time, but in comparison the freezing point of the sap of *Halicystis* kept in a flow of this sea water always has a slightly greater freezing point depression. For example, in one case the freezing point of the sea water was -2.030°C and the freezing point of the sap of cells kept in it was -2.059°C. The usual difference of freezing point between sea water and the sap of *Valonia* is considerably greater than this. For example, in one case when the freezing point of the sea water in contact with the cells was -2.021°C the freezing point of the *Valonia* sap was -2.150°C. Qualitatively this difference can be observed in the turgidity of the cells. *Halicystis* cells are invariably soft and rubbery to the touch, *Valonia* cells are firm and hard. In the case of *Valonia* we were unable to determine by calculation if the early more rapid entrance of

<sup>4</sup> The osmotic concentration is unity when the freezing point depression equals 1.864 i.e. the freezing point depression of 1 mole of non electrolyte dissolved in 1 liter of aqueous solution.

water immediately after impalement represents the more rapid entrance of water than electrolyte with a corresponding adjustment of the osmotic concentration of the sap to that of the sea water. But it seems probable that this is the case.

In the case of *Halocystis* the difference in osmotic concentration between sap and sea water is much less. The osmotic concentration of sap is  $2.059 - 1.864 = 1.1046$  M. Now in the case of cell 17 (Fig. 3) the initial period of rapid rise lasted about 92 minutes during which time the volume increased by 0.0088 cc, but we may suppose that only part of this was pure water, since electrolytes could enter also and it is a reasonable assumption that the rate of electrolyte entrance might be constant throughout the experiment. Therefore part of the increase in volume may be considered as the addition of undiluted sap to the volume. Interpolating from the straight part of the curve which apparently represents the increase in volume without dilution we find that in 92 minutes the amount of undiluted sap entering would be approximately 0.0045 cc. The original volume of the cell was 0.348 cc. Hence the calculated osmotic concentration should be approximately 1.0913 M. But the osmotic concentration of the sea water in contact with the cell was approximately  $2.030 - 1.864 = 1.0891$  M, a negligible difference.

On the other hand, similar calculations in the case of cell 15 gave for the probable osmotic concentration of the sap at the end of the rapid initial volume increase 1.0986 which suggests that the complete osmotic adjustment did not occur in this case. The evidence in this connection from halide analysis is also not decisive because the calculated change of halide concentration corresponding to complete osmotic adjustment is smaller than the natural variations in halide concentration among normal cells.

On the whole, therefore, the evidence while pointing to some osmotic adjustment does not support decisively the view that complete osmotic equality is attained. From a theoretical viewpoint, however, we think it desirable to assume that osmotic equality does occur on impalement.

We shall assume at first, that, just as in the case of *Valonia*, the entrance of sodium and potassium occurs by the diffusion of  $\text{NaX}$  and  $\text{KX}$  in the aqueous layer of the protoplasm, where  $X$  is the anion

of a weak acid  $HX$  elaborated by the protoplasm. In the present case, since the concentration of potassium in the sap is very small, we shall discuss sodium entrance only.<sup>5</sup> In a previous paper we have suggested that the flux of  $M$  ( $M$  may be sodium or potassium) across a unit area of protoplasm of unit thickness is given by the equation

$$\frac{dM}{dt} = D^{MX} \{ [M\lambda]_{epo} - [MX]_{epi} \}$$

and this with suitable simplifications reduces to

$$\frac{dM}{dt} = k_{coll} D^{MX} \{ [M]_o (OH)_o - [M] (OH)_i \}$$

where

$$k_{coll} = \frac{f_o^{Na} f_{epo}^{HX} S_{cop}^{NaX} [H\lambda]_{epo}}{f_{cop}^{NaX} \frac{HX}{S_{cop}} \frac{NaX}{k_{hydrolysis}}}$$

In these equations  $D$  is the diffusion constant,  $S$  is the partition coefficient,  $f$  is the activity coefficient, and  $k_{hydrolysis}^{NaX}$  is the thermodynamic hydrolysis constant of the reaction



Round brackets indicate activities and square brackets concentrations. The subscripts  $o$  and  $i$  refer to the bulk of the sap and sea water respectively, and the subscripts  $epo$  and  $cop$  refer to a pair of adjacent unstirred layers in the protoplasm and sea water respectively in which the solute and solvents are in equilibrium across the interface  $epi$  and  $cop$  refer to similar layers at the sap protoplasm interface.

In the derivation of this equation it was assumed that corresponding activity coefficients and partition coefficients in the sap and sea water are equal.  $HX$  is also considered to be distributed equally throughout the protoplasm.

The rate of entrance of water is given by the flux of water across the non aqueous protoplasm, viz

$$\frac{dH_2O}{dt} = D^{H_2O} \{ [H_2O]_{ep} - [H_2O]_{epi} \}$$

<sup>5</sup> The situation is complicated by the fact that halide is entering the cell simultaneously. A mechanism whereby halide may enter will be discussed elsewhere.



Now the entrance of water and electrolyte goes on under steady state conditions. Hence the ratio of the rates of entrance of electrolyte to water remains constant while only the volume of the sap increases. There is good evidence that the rate of entrance of water takes place much more rapidly than the rate of electrolyte entrance. Thus, as we showed in a previous paper, even when the sea water is diluted extensively the adjustment of the osmotic pressure between the sea water and the sap of an impaled *Halcystis* cell takes place almost entirely by entrance of water into the sap and hardly at all by loss of electrolyte <sup>6</sup>

If this is so then in the impaled cell we should expect the osmotic concentration of the sap at the steady state to be very close to that of the sea water, for when the cell takes in electrolyte and thereby raises the osmotic concentration by a small amount water can enter at once, since there is no volume restriction due to the cell wall. This would tend to abolish the osmotic gradient.

When the cell is intact we expect a different steady state concentration in the sap, for then water can enter only when the cell wall grows and so is able to enclose more space. Hence the rate of water entrance is decreased, and in order to decrease the rate of electrolyte entrance  $\{[Na]_s(OH)_s - [Na]_o(OH)_o\}$  must decrease. This can only happen by  $[Na]_s$  increasing since the other concentrations are fairly well fixed. We may note that the situation is somewhat different from the situation in Osterhout's guaiacol model, where the rate of uptake of water is increased as the concentration of electrolyte in the inner phase increases. In the present case the rate of uptake of water cannot increase as the concentration of the electrolyte in the sap increases, because of the cell wall restriction. Consequently all the adjustment to the new steady state depends on  $[Na]_s$  increasing. We need not predict at what point the steady state will be established but it is clear that it must be much higher than 0.6 M for  $(OH)_s$  is at least 100 times  $(OH)_o$ , hence  $[Na]_s$  may increase many fold without decreasing the gradient appreciably <sup>7</sup>

<sup>6</sup> Jacques, A. G., *J. Gen. Physiol.*, 1938-39, 22, 743

<sup>7</sup> Let us consider a cell containing 1 ml of sap. In the impaled cell the cell sap gains in about 10 days (10 per cent increase in volume per day, the concentration of electrolyte being 0.6 M) 1 ml of water and 0.6 millimole of electrolyte. Now suppose an impaled cell could be suddenly made intact. The cell would

It was because practically the same steady state was obtained in both intact and impaled *Valonia* cells, even though there was a 15 fold change in the rate of entrance of water and electrolyte, that we were led to suggest a regulatory mechanism which operates by changing the partition and diffusion coefficients of  $MX$  in the non aqueous protoplasm. It was suggested that the mechanism operates about as follows. In the intact cell a little electrolyte enters thereby raising the osmotic concentration of the sap. As a result water enters and stretches the cellulose wall to the limit. Electrolyte continues to enter but water cannot until more cellulose is produced. Then as the osmotic pressure of the sap increases, water is withdrawn from the protoplasm. This does not require any change in the volume (sap + protoplasm) inside the cellulose envelope, but it does change the properties of the non aqueous protoplasm, by lowering the partition coefficient of  $MX$ , so that  $\{MX_{sap} - MX_{pi}\}$  is decreased.<sup>8</sup> At the same time the viscosity of the protoplasm is increased and  $D^{MX}$  is decreased.

On the whole we think that the same explanation may apply in the present case. Before discussing it let us consider an alternative mechanism by which sodium is assumed to enter the cell as sodium chloride.

According to the analysis of Bermuda sea water and *Halcyris* sap<sup>9</sup>  $[Na]_o = 0.498$  M,  $[Cl]_o = 0.580$  M,  $[Na]_i = 0.557$  M, and  $[Cl]_i = 0.603$  M. Since the ionic strengths of the sap and sea water are quite close to each other the activity coefficients may be taken as equal.<sup>10</sup>

now gain 1 ml. of water in about 100 days under these conditions, and if the rate of entrance of electrolyte remained unchanged it would gain 6 millimoles of electrolyte in the same time and the concentration would become  $6.6 - 2 = 3.3$  M. But if the concentration were 3.3 M the gradient would be reduced from approximately  $5.94 \times 10^{-7}$  to  $5.67 \times 10^{-7}$ . This very approximate calculation is made as follows  $[Na] = 0.6$  M,  $[OH] = 10^{-6}$  M,  $[Na]_i = 0.6$  M,  $[OH]_i = 10^{-8}$  M  $[Na] \times [OH] = 6 \times 10^{-7}$ , and  $[Na]_i \times [OH]_i = 0.06 \times 10^{-7}$ , whence the difference is  $5.94 \times 10^{-7}$ . When  $[Na]_i$  is taken as 3.3 M,  $[Na]_i \times [OH]_i = 0.33 \times 10^{-7}$  and the difference becomes  $5.67 \times 10^{-7}$ . That is, it would decrease less than 10 per cent. We need not attempt to fix the steady state concentration more closely.

<sup>8</sup> Regarding *epo*, *eop*, *epi* and *eip* see p. 765.

<sup>9</sup> Osterhout, W. J. V., *J. Gen. Physiol.* 1922-23, 5, 225. Blinks, L. R., and Jacques, A. G., *J. Gen. Physiol.* 1929-30, 13, 733.

<sup>10</sup> The ionic strength of Bermuda sea water, according to the calculation of

Hence the chemical potential of sodium chloride is greater in the sap than in the sea water. Consequently NaCl as such cannot diffuse from the sea water to the sap without the cell supplying energy. If sodium chloride diffuses as such from the sea water to the sap we may write for the flux of NaCl

$$\frac{d[\text{NaCl}]}{dt} = \frac{D^{\text{NaCl}}}{h} \{[\text{NaCl}]_{epo} - [\text{NaCl}]_{epi}\} \quad (1)$$

or

$$= \frac{D^{\text{NaCl}}}{h} \{S_{cop}[\text{Na}]_o[\text{Cl}]_o - S_{cip}[\text{Na}]_i[\text{Cl}]_i\}^* \quad (2)$$

\* The assumption and steps by which we arrive at equations similar to the present one have been applied in several forms in another paper (Jacques, A. G., *J. Gen. Physiol.*, 1938-39, 22, 147) and they need not be considered in detail here.

In order for the right hand term of the gradient to become less than the left hand in *Halicystis*  $S_{cip}$  must be less than  $S_{cop}$ . But in any diffusion system to which no energy is supplied  $S_{cop}$  and  $S_{cip}$  must obviously come closer together as  $[\text{Na}]_o[\text{Cl}]_o$  and  $[\text{Na}]_i[\text{Cl}]_i$  come closer together until when these products are equal the partition coefficients are also equal. In a system such as *Halicystis* it seems not impossible that the required energy may be available.

Suppose the energy of the cell were directed to the production of a substance which depresses the solubility of sodium chloride in the non-aqueous phases. This would lower the partition coefficients. But in order for such a substance to lower  $S_{cip}$  differentially it would be necessary for it to be present in higher concentrations at the sap-protoplasm interface than at the sea water-protoplasm interface. This seems possible, for example, if the substance is produced only at the sap-protoplasm interface and has a low rate of diffusion in the non-aqueous protoplasm. Thus we can imagine a "steady state"

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Zscheile (Zscheile, F. P., Jr., *Protoplasma*, 1930, 11, 481) is 0.7212, and the ionic strength of *Halicystis* sap by our calculation is 0.6326. By reference to the data of Harned (Harned, H. S., in Taylor, H. S., *A treatise on physical chemistry*, New York, D. Van Nostrand Company, Inc., 2nd edition, 1931, 1, 772) we see that between these two ionic strengths the activity coefficient of sodium chloride changes hardly at all.

in which the "depressant" agent is being produced rapidly at the sap-protoplasm interface and is diffusing slowly to the sea water protoplasm interface, so that a rather steep but constant gradient of the substance is set up across the protoplasm  $S_{n,p}$  then remains continuously less than  $S_{s,p}$  and the flux of NaCl is inward in spite of the fact that  $[Na]_i, [Cl]_i > [Na], [Cl]$

The part played by the aqueous phases may be important. Since the depressant agent is able to displace the polar sodium chloride from the non polar protoplasm, it must itself be relatively more non polar. Hence its partition coefficient must be high. At the sap protoplasm interface we have the relationship

$$S_{s,p}[a]_{s,p} = [a]_{s,i}$$

where  $a$  is the depressant agent. In the steady state it seems likely that  $[a]_{n,p} = [a]_i$  (where  $[a]_i$  is the concentration in the body of the sap) because the volume of the sap is limited.

At the sea water protoplasm interface, however,  $[a]_{s,p} > [a]_i$  since the sea water is not limited in volume. Hence there is a loss of the agent to the sea water, and in the steady state this may take place at such a rate that the decrease in the *epo* layer is just made up by the diffusion of the agent from the sap protoplasm interface. This steady loss is important since if the depressant were produced continuously and did not escape from the protoplasm no steady state would be possible. Instead the concentration of the depressant would eventually become equal at the two interfaces when, of course, its differential effect on the partition coefficients of sodium chloride at sea water and sap interfaces would disappear.

Osterhout<sup>11</sup> has suggested that the protoplasm of such large plant cells as *Halocystis* consists of two non aqueous layers,  $X$  and  $Y$ , adjacent to the sea water and sap respectively, and  $W$ , an aqueous layer, between them. It might be supposed that if the  $X$  and  $Y$  layers have different properties  $S_{s,p}$  and  $S_{n,p}$  must necessarily be different, and that accumulation of sodium chloride as NaCl could occur on this account alone. It is easy to see that this could not be so thermodynamically. As a matter of fact the system does not

<sup>11</sup> Osterhout W J V *Ergebn Physiol* 1933, 35, 1014

differ fundamentally from a system with only one non-aqueous layer separating sea water and the sap

Suppose we have a three layer system in which the diffusion coefficients are different and suppose the thicknesses of the layers are  $h_x$ ,  $h_w$ , and  $h_y$ . The total thickness of the system is small so that "steady state diffusion" prevails. The flux in all parts of the system is therefore the same. Jacobs<sup>12</sup> has shown for a two layer system of this type that the rate of transfer of solute is proportional to the gradient across the whole system. The separate gradients across each layer need not be considered. Extending Jacobs' reasoning to our three layer system we have for the flux across a unit area

$$\frac{d[\text{NaCl}]}{dt} = \frac{D_x D_w D_y}{D_y D_w h_x + D_y D_x h_w + D_x D_w h_y} \{[\text{NaCl}]_{epi} - [\text{NaCl}]_{epo}\} \quad (3)$$

which is very similar to the equation (1) set up for the flux in a single layer

It might be suggested that the supposed diffusion of the depressant from the sap to the sea water resembles the movement of an ionogenic "diffusing agent" which Teorell<sup>13</sup> has shown is capable of bringing about accumulation in non-living systems. The resemblance is superficial since the depressant is not supposed to be ionogenic and hence cannot furnish the ions of different mobility required to produce the electrical stress which according to Teorell's theory is the force which brings about the redistribution of other ions in the system.

In an average *Halcystis* cell  $[\text{Na}]_o$ ,  $[\text{Cl}]_o = 0.289$  and  $[\text{Na}]_i$ ,  $[\text{Cl}]_i = 0.336$ . Hence if the ratio  $S_{e,p} - S_{ep}$  could be maintained at a value slightly greater than 1.16 sodium could enter the cell as sodium chloride. Let us suppose for the moment that this condition is met in the intact cell. When the cell is impaled the rate increases about 10-fold hence if  $D^{\text{NaCl}}$  remains unchanged  $S_{ep}$ ,  $[\text{Na}]_o$ ,  $[\text{Cl}]_o - S_{e,p}$ ,  $[\text{Na}]_i$ ,  $[\text{Cl}]_i$  must increase 10-fold. Since there is only a very slight decrease of  $[\text{Na}]_i$ ,  $[\text{Cl}]_i$  in the impaled cell the change must be effected through the partition coefficients. However, it is difficult to see

<sup>12</sup> Jacobs, M. H., *Ergebn Biol*, 1935, 12, 70

<sup>13</sup> Teorell, T., *Proc Nat Acad Sc*, 1935, 21, 152, *J Gen Physiol*, 1937-38, 21, 107

how the results we observe could come about through the depressant  $a$ . There is no reason to suppose that its rate of production would be increased by impalement. But since the volume of the sap increases faster in the impaled cell, the loss of  $a$  to the sap will be greater, hence  $[a]_{epi}$  will be smaller.

This effect may be small and the increase in  $S_{ep}$  due to the decrease in the concentration of the agent may in part be compensated by the smaller concentration of  $a$  in the *epo* layer which will cause  $S_{ep}$  to increase. The net effect on the partition coefficient ratio will probably be nearly zero. And in any case there seems to be no possibility that it can be so increased by the depressant that the gradient term increases 10 fold.

Thus even if sodium diffuses in by the mechanism suggested above it seems necessary to assume that there is an additional mechanism whereby the gradient is altered when the cell is impaled.

As pointed out above we assume that the additional mechanism operates by withdrawing water from the non aqueous protoplasm to reduce both  $S_p$  and  $S_{ep}$ , and at the same time by increasing the viscosity of the non aqueous phase to reduce the diffusion coefficients. According to this view in the intact cell some of the energy of the cell is directed towards decreasing the rate of uptake of electrolyte. When the cell is impaled the energy is no longer expended in this way and without the need for an increase in the energy output of the plant the rate of uptake of electrolyte can increase.<sup>14</sup>

However, recent results of Blinks, Darsie, and Skow<sup>15</sup> on the Pacific Coast *Halocystis ovalis* indicate that on impalement the cell consumes oxygen at a more rapid rate than normal and this keeps up for some time "even for several days." That is to say on impalement the en

<sup>14</sup> An alternative mechanism for keeping the osmotic concentration of the cell sap from increasing much above that of the sea water in the intact cell has been suggested to us by Dr T. Shedlovsky. He suggests that when the cell is stretched to the limit the protoplasm ruptures momentarily and as a result sap escapes and the vacuole volume decreases. Then the rupture heals and as the cellulose wall is not now stretched to the limit water can enter to stretch it, thereby reducing the osmotic concentration. This process is supposed to be repeated an indefinite number of times.

<sup>15</sup> Blinks, L. R., Darsie, M. L., Jr., and Skow, R. K., *J. Gen. Physiol.*, 1938-39, 22, 255.

ergy output of the cell is increased. Hence in contrast to the view expressed above it might be suggested that the increased rate of uptake of electrolyte is in some way associated with this. However, although in the present paper, the longest time during which the rate was followed was 5 days, in a forthcoming paper<sup>16</sup> we shall describe experiments in which the accelerated rate of uptake over that of the intact cell continued for periods up to 15 days and it scarcely seems likely that the accelerated rate of oxygen consumption would continue so long.

Possibly, however, the more rapid non-linear rate of increase in volume during the first few hours is associated with increased energy output. But it seems just as likely that it is associated with the partial dissipation of the excess osmotic energy of the sap over that of the sea water.

Since in the case of the impaled *Valonia* cell the experiments were terminated in 48 hours or less, two questions arise. First, does impalement result in increased energy output for several days in *Valonia* as it does in *Halicystis ovalis*, and second, is the increased rate of uptake of electrolyte associated in any way with increased energy output? We think that the last question should be answered negatively, for the reason that *Valonia* and *Halicystis Osterhoutii* on impalement behave qualitatively and quantitatively nearly alike, and, as pointed out already, in the latter case it seems unlikely that the increased energy output would persist as long as the increased rate of uptake lasted.

In the case of *Valonia*, measurements on the sap made in two cases after 48 hours of increased electrolyte uptake due to impalement, failed to show any definite change in the pH. Yet if the cell were to continue to respire more rapidly on impalement we might possibly expect to find the pH of the sap lower than normal.

Between the two alternatives entrance as NaX or as NaCl by the aid of a depressant, there is little to choose as far as the evidence goes, but in the case of certain plants such as *Nitella*, where the osmotic pressure and the concentrations of most of the ions in the sap are greater than in the surrounding medium, it seems possible that favor-

<sup>16</sup> Jacques, A. G., *J. Gen. Physiol.*, 1939-40, 23, in press.

able gradients may depend on substances present in larger concentration at the sap protoplasm interface, which depress the solubility of the salts in the non aqueous phases

#### SUMMARY

When cells of *Halocystis* are impaled on a capillary so that space is provided into which the sap can migrate, the rate of entrance of water and of electrolyte is increased about 10 fold. In impaled *Valonia* cells the rate is increased about 15 fold.

After a relatively rapid non linear rate of increase of sap volume immediately after impalement (which may possibly represent the partial dissipation of the difference of the osmotic energy between intact and impaled cells) the volume increases at a linear rate, apparently indefinitely.

Since the halide concentration of the sap at the end of the experiment is (within the limits of natural variation) the same as in the intact cell, we conclude that electrolyte also enters the sap about 10 times as fast as in the intact cell.

As in the case of *Valonia* we conclude that there is a mechanism whereby in the intact cell the osmotic concentration of the sap is prevented from greatly exceeding that of the sea water. This may be associated with the state of hydration of the non aqueous protoplasmic surfaces.





# THE PORPHYROPSIN VISUAL SYSTEM

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Certain fishes have evolved a visual system different from that common to all other types of vertebrate. In place of the rose colored rhodopsin, their rods contain a purple photolabile pigment which I have suggested be called porphyropsin (Wald, 1937 a). This peculiarity was first noted in a number of fresh water fishes by Kühne and Sewall (1880), and later was confirmed spectrophotometrically by Kottgen and Abelsdorff (1896).

Following the work of the latter authors it has been believed that fish retinas in general contain porphyropsin. Recently, however, a number of marine fishes have been shown to possess visual systems identical with those of frogs and mammals, in which rhodopsin participates in a retinal cycle with the carotenoids retinene and vitamin A (Wald, 1936-37, 1937-38).

This suggested the curious possibility that porphyropsin is restricted to fresh water fishes. And in fact a preliminary survey indicates that the retinas of most permanently marine fishes contain rhodopsin, those of permanently fresh water fishes porphyropsin, while those of euryhaline<sup>1</sup> fishes contain either predominantly or exclusively that photopigment ordinarily associated with the environment in which the fish is spawned (Wald, 1938-39).

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<sup>1</sup> It has been found convenient to divide these fishes into four classes: two groups of stenohaline fishes restricted permanently to marine or fresh water environments and two groups of euryhaline fishes capable of existence in a wide range of salinities: those spawned in fresh water (anadromous) and those spawned in the sea (catadromous).

The present paper is concerned with the chemistry of the "pure" porphyropsin system as found in permanently fresh-water and some anadromous fishes, specifically with the behavior of porphyropsin in the retina and in aqueous solution, and its interaction with carotenoids in the retinal cycle. In this work I have followed closely the design of previous experiments on the rhodopsin system in marine fishes (Wald, 1936-37, 1937-38), in order to demonstrate clearly that both systems, though distinctly different in composition, are remarkably parallel in behavior. To aid in this comparison, I have included in the present paper new observations on the marine scup and on two members of the previously unexplored group of marine elasmobranchs.

### Methods

*Porphyropsin Solutions* —Retinas of dark adapted fishes were prepared in dim red light, as free as possible of the underlying pigmented epithelium and choroid layer. After soaking for about 3 hours in a 4 per cent solution of aluminum potassium sulfate (alum), they were washed twice in distilled water and twice in phosphate buffer solutions, usually neutral. Finally they were ground in 2 per cent aqueous digitonin, and allowed to leech at room temperature overnight. The mixtures were centrifuged for about 20 minutes at about 3000 R P M and the clear solutions stored at 2°C. Generally these solutions were recentrifuged just before their spectra were recorded to dispose of colorless precipitates which sometimes developed during storage.

*Antimony Chloride Tests* —Retinal extracts in dry chloroform were tested by mixing with saturated solutions of antimony trichloride in chloroform in the proportion 0.3 cc. of extract to 3.2 cc. of reagent. Spectra of these mixtures were recorded immediately, since the test color fades and changes qualitatively within 10 to 15 minutes.

*Spectrophotometry* —Spectra in the visible region were measured with the recording photoelectric spectrophotometer of A. C. Hardy at the Color Measurements Laboratory of the Massachusetts Institute of Technology (Hardy, 1935). The special advantages of this instrument for the type of work with which we are concerned have already been indicated (Wald, 1937-38). Figs. 1, 2, 3, and 6 are direct recordings drawn by the instrument itself, Fig. 8 is a tracing from such a record. In each case the difference in absorption between the test solution and a blank of pure solvent of equal depth was measured. The absorption is recorded either as percentage of incident light absorbed ( $1-I/I_0$ ), or as extinction (optical density),  $\log I_0/I$ , in which  $I_0$  is the incident and  $I$  the transmitted intensity.

Absorption spectra in the ultra-violet were measured with a Hilger Intermediate quartz spectrograph, equipped with a Spekker Photometer, and using a tungsten spark as source.

*Animals*—The fishes which have been examined include the fresh water calico bass (*Pomoxis sparoides*), carp (*Cyprinus carpio*), blue gill (*Lepomis palidus*), and pickerel (*Esox reticulatus*), the anadromous white perch (*Morone americana*), and the marine scup (*Stenotomus chrysops*) smooth dogfish (*Galeorhinus laevis*), and spiny dogfish (*Squalus acanthias*)

### *The Retinal Cycle*

Isolated retinas of dark adapted fresh water fishes are deep purple in color. On exposure to bright daylight they bleach almost instantly to a russet hue, which at 0°C is maintained for hours, even in bright light. This russet material represents the end product of the photochemical reaction.

At room temperatures in moderate light the initial russet color fades slowly to faintest greenish-yellow, virtually to colorlessness. This process occupies about  $\frac{3}{4}$  of an hour at 25°C. It occurs in the dark as well as in the light, and possesses a high temperature coefficient. It is an ordinary thermal reaction.

A russet retina replaced in darkness at room temperatures, in addition to fading, also regenerates some porphyropsin, by rough visual estimate perhaps  $\frac{1}{3}$  of its original content. Retinas in intermediate stages of fading regenerate less porphyropsin, in amounts roughly proportional to their content of russet products. The completely faded retina does not synthesize porphyropsin at all.

It may be concluded that in the isolated retina light bleaches porphyropsin to a russet pigment, which is removed by thermal processes in two directions: (a) by reversion to porphyropsin, and (b) by irreversible formation of a faint yellow product.

Bleaching of the isolated retina ends, therefore, in the faintly yellow, almost colorless condition. The retinas of living fish which had been exposed to direct sunlight for 15 minutes and then dissected out either in the light or immediately on removal to darkness, possess the same virtually colorless appearance. Yet *in vivo* such retinas rapidly regain their original content of porphyropsin in darkness. Though the isolated retina cannot synthesize porphyropsin from the faintly yellow product, it is clear that this process occurs readily in the intact animal.

The porphyropsin, like the rhodopsin system, therefore, constitutes a cycle within a cycle. Porphyropsin bleaches in the light reversibly to a russet pigment. This in turn fades irreversibly to a light yellow

material, which in the intact eye, though not in the isolated retina, is re-synthesized to porphyropsin

*Vitamin A<sub>2</sub> and Retinene<sub>2</sub>*

Rhodopsin is a conjugated protein bearing a carotenoid prosthetic group. The color changes which characterize the rhodopsin cycle depend primarily upon alterations in its carotenoid components. The remarkably parallel gross behavior of porphyropsin in the retina suggests that it may be some simple chemical variant of rhodopsin, involving the carotenoid or the protein residue, or the linkage between them. The first of these alternatives proves to be correct, and provides a sufficient basis for all the observations. The porphyropsin cycle, though identical in form with that of rhodopsin, contains new carotenoids in the positions of retinene and vitamin A.

This situation is revealed in a simple procedure originally applied to marine fishes (Wald, 1936-37, see particularly Fig 1). Its application to the smooth dogfish, a marine elasmobranch, is shown in Fig 1, and to the permanently fresh-water calico bass and pickerel in Figs 2 and 3.

Right and left retinas of several dark adapted fish are isolated separately in dim red light, so that two symmetrical sets of retinas from the same animals result.

One group of these is centrifuged, the clear Ringer's solution decanted, and the tissue extracted by shaking for 20 minutes by machine *in darkness* with benzine (petroleum ether, boiling range 30 to 60°C). This treatment does not disturb the retinal photopigments. The mixture is centrifuged, the benzine extract poured off, and transferred to 0.3 cc of chloroform. It forms a virtually colorless solution. The whole of this solution is mixed with 3.2 cc of antimony chloride and the absorption spectrum of the faintly blue product measured immediately. Marine fishes yield in this test a low absorption band, maximal at about 618 mμ, showing the dark adapted retina to contain a small quantity of vitamin A (Fig 1 a). Extracts from fresh-water fishes yield a similar band, its maximum, however, lies at 696 mμ (Figs 2 and 3, curves a).

Following extraction in the dark with benzine, the retinal tissue is still deeply colored with photopigment. On exposure to bright light

it bleaches quickly to orange or russet. It is immediately re extracted with benzine, exactly as before. The extract, transferred to 0.3 cc

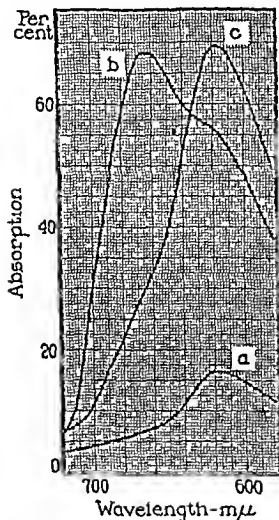


FIG 1 Antimony chloride reactions with benzine extracts of retinas of the smooth dogfish (a) Dark adapted retinas yield a small amount of the 618  $m\mu$  chromogen, vitamin A (b) Irradiated retinas, a large quantity of the 664  $m\mu$  chromogen, retinene has been liberated (c) Completely faded retinas, practically all retinene has been converted to vitamin A

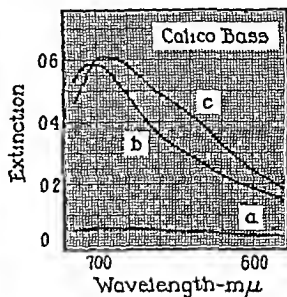


FIG 2 Antimony chloride reactions with benzine extracts of retinas of the calico bass (a) The dark adapted retina contains a trace of the 696  $m\mu$  chromogen, vitamin A<sub>2</sub> (b) Irradiated retinas, a large amount of the 704  $m\mu$  chromogen, retinene<sub>2</sub> has been liberated (c) Faded retinas, the retinene<sub>2</sub> has been converted to vitamin A<sub>2</sub>

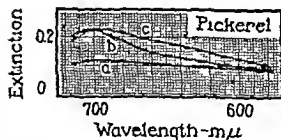


FIG 3 Antimony chloride reactions with benzine extracts of pickerel retinas. Otherwise as in Fig 2

chloroform, is golden in color. Tested as before with antimony chloride, marine fish extracts yield a high band, maximal at 664  $m\mu$ , the irradiation of rhodopsin has liberated a large quantity of

retinene (Fig 1 *b*) From fresh-water fishes a parallel result is obtained, but in this case the antimony chloride maximum lies at 703–706  $m\mu$  (Figs 2 and 3, curves *b*)

The symmetrical set of retinas is exposed to bright light in Ringer's solution and is left at room temperatures in moderate light for about an hour During this period the retinas fade almost to colorlessness Finally they are collected by centrifuging and extracted with benzine exactly as were the others The extracts, transferred to 0.3 cc chloroform, are virtually colorless Tested with antimony chloride, they yield in the case of marine fishes a high band at about 618  $m\mu$  with little or no specific absorption at 664  $m\mu$  The fading process has transformed the retinene liberated on irradiation quantitatively to vitamin A (Fig 1 *c*) Fresh-water fishes yield a parallel result, but the antimony chloride band of the final product lies at 696  $m\mu$  (Figs 2 and 3, curves *c*)

This final state is qualitatively identical with that obtained by high light adaptation of the living fish, *in vivo* it reverts in darkness to the original condition represented by curves *a* of Figs 1 to 3 The transitions revealed in this procedure therefore characterize in the intact animal three stages in the retinal cycle The cycle is identical in form in both fresh-water and marine fishes, but in the former a 703–706  $m\mu$  chromogen replaces retinene, and a 696  $m\mu$  chromogen vitamin A No trace of either vitamin A or retinene has yet been found in a fresh-water fish retina

The data of Fig 1 were obtained from four smooth dogfish about 25 inches in length Each curve, therefore, was derived from the whole extract of four retinas Similarly the data of Figs 2 and 3 were obtained from eight calico bass about 8 inches in length, and from eight pickerel 10 inches in length

Similar experiments have been performed with the anadromous white perch (*cf* Wald, 1937 *a*) and with the carp, with results essentially identical with those of Figs 2 and 3

These observations were first reported early in 1937 Almost simultaneously Lederer and Rosanova (1937) reported that though the liver oils of a number of Russian marine fishes yield the familiar antimony chloride band of vitamin A, those of most fresh-water fishes which were examined yield instead an "abnormal reaction" consisting of a dominant band at about 690  $m\mu$  It was clear that the substance

which replaces vitamin A in the fresh water fish retina may do so also in the liver

The retinal activity of the new substance demonstrated its vitamin nature, while its discovery in liver oils provided a rich source for its further investigation. This has proceeded with great rapidity. The 696  $m\mu$  chromogen was shown to possess a direct absorption band in ethanol at 345–350  $m\mu$ . It is probably a homologue of vitamin A, containing an added ethylene group and the empirical formula  $C_{22}H_{32}O$  (Gillam, Heilbron, Jones, and Lederer, 1938). A pike perch liver oil which probably contained little if any vitamin A displayed growth promoting activity in rats about equal to that of a halibut liver oil of the same Lovibond value (Gillam *et al.*). Rats and frogs, which normally contain little or no 696  $m\mu$  chromogen, accumulate this substance in their livers when fed fresh water fish liver oil concentrates (Lederer and Rathmann, 1938). A similar accumulation may occur naturally in certain fish-eating mammals (Gillam, 1938). The distribution of vitamin A and the 696  $m\mu$  chromogen in a wide variety of animals and tissues has been reported in the papers cited, by Edisbury, Morton, Simpkins, and Lovern (1938), and by Wald (1938–39).

Since the 696  $m\mu$  chromogen replaces vitamin A in the retinal cycle of fresh water fishes, Edisbury, Morton, and Simpkins (1937) have suggested that it be called *vitamin A<sub>2</sub>*.<sup>2</sup> The retinene analogue in these fishes may correspondingly be called *retinene<sub>2</sub>*. These carotenoid substances with porphyropsin constitute the known components of the fresh water fish visual system.

The direct spectra of rhodopsin and porphyropsin in aqueous solution and of the retinenes and vitamins A in chloroform, all brought to the same height to facilitate comparison, are shown in Fig. 4. They were derived from crude untreated preparations resulting from precisely parallel procedures in both systems. The photopigment solutions were aqueous digitonin extracts of alum treated dark adapted retinas. The retinenes were obtained by immediate benzene extraction of irradiated retinas, from which free vitamins A had previously been removed by benzene extraction in darkness. The vitamins A

<sup>2</sup> Cf. footnote in Wald, 1938–39, page 392



were obtained by simple benzine extraction of completely bleached retinas. The solutions of retinenes and vitamins A were therefore precisely similar to those which yielded the antimony chloride tests shown in curves *b* and *c* of Figs 1 to 3. It is clear that the direct absorption spectra throughout both systems are single-banded, and maintain a consistent parallelism. The significance of these observations is discussed below.

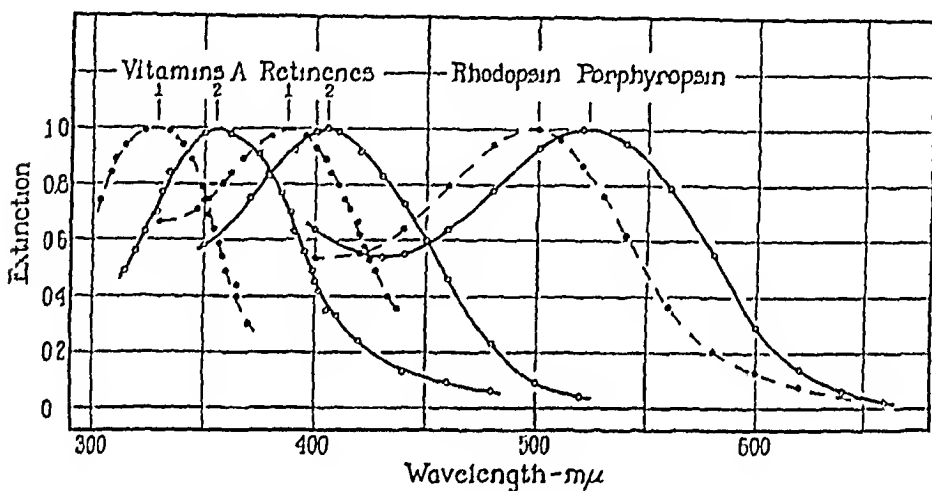


FIG 4 Direct spectra of retinal extracts of the calico bass (circles, solid lines) and of the marine scup (broken lines). The rhodopsin and porphyropsin preparations are digitonin extracts of alum-treated retinas, measured in 1 per cent aqueous digitonin solution. The retinene preparations are crude untreated benzene extracts of irradiated retinas, measured in chloroform solution, the vitamin A preparations are similar solutions of extracts of completely faded retinas. All maxima have been brought to the same height to facilitate comparison.

Vitamin A<sub>2</sub> is a light yellow pigment found in the non-saponifiable fraction of tissue oils. It partitions about equally between 90 per cent methanol and benzine, and is about 70 per cent hypophasic when shaken with 95 per cent methanol and benzine. In chloroform it displays a broad absorption band maximal at about 355 mμ, accompanied in all preparations so far examined by a low band at about 290 mμ. Tested with antimony chloride it yields a high band at 696 mμ, accompanied by a small, broad hump at about 645 mμ. In crude liver oils the 696 mμ band may be displaced to positions as low as 685 mμ.

It is not yet known whether the satellite direct absorption at about 290  $m\mu$  and in the antimony chloride test at 645  $m\mu$  are due to vitamin A<sub>2</sub> itself or to a contaminant

Retinene<sub>2</sub> is deep yellow in color. It is non saponifiable and partitions principally epiphasically between 90 per cent methanol and benzene. In chloroform it possesses a broad band maximal at about 405  $m\mu$ , and with antimony chloride the best preparations yield a band at 706  $m\mu$ . In other preparations the antimony chloride band may be displaced as low as 702  $m\mu$ . Retinene<sub>2</sub>, like retinene, appears to change color markedly with hydrogen ion concentration, tending toward very light yellow in alkaline and deep orange in acid solution.

### *Porphyropsin in Aqueous Solution*

Porphyropsin, like rhodopsin, may be extracted into aqueous solutions of bile salts or digitonin. Such solutions are deep purple in color. A collection of spectra of porphyropsin preparations is shown in Fig 5, and for comparison the spectrum of a rhodopsin solution from the spiny dogfish, prepared in the same manner. Above 470  $m\mu$  the porphyropsin spectra form a homogeneous group, maximal at  $522 \pm 2$   $m\mu$ . Below 470  $m\mu$  they diverge, due to the presence of varying amounts of impurities. A minimum has always appeared in these spectra, varying in position with the degree of purity, but approaching a low wavelength limit at about 430  $m\mu$  in the best preparations. It is not yet known whether this is characteristic of porphyropsin itself, or whether it is an artefact, and the rise in absorption below 430  $m\mu$  due to contaminants. The porphyropsin band is wider than that of rhodopsin, its displacement varying from about 20  $m\mu$  on the low wavelength side of the maximum to about 30  $m\mu$  on the high wavelength side.

These spectra are not to be confused with the reputed absorption spectra of fish photopigments measured by Kottgen and Ahelsdorff (1896) and since grown familiar in the literature. The latter consist of a single broad band, falling almost symmetrically away from a maximum at about 540  $m\mu$  to negligible absorptions at about 440 and 700  $m\mu$ . Kottgen and Ahelsdorff reported in these observations only the differences in absorption between unbleached and bleached porphyropsin solutions ("difference spectra"). These should be

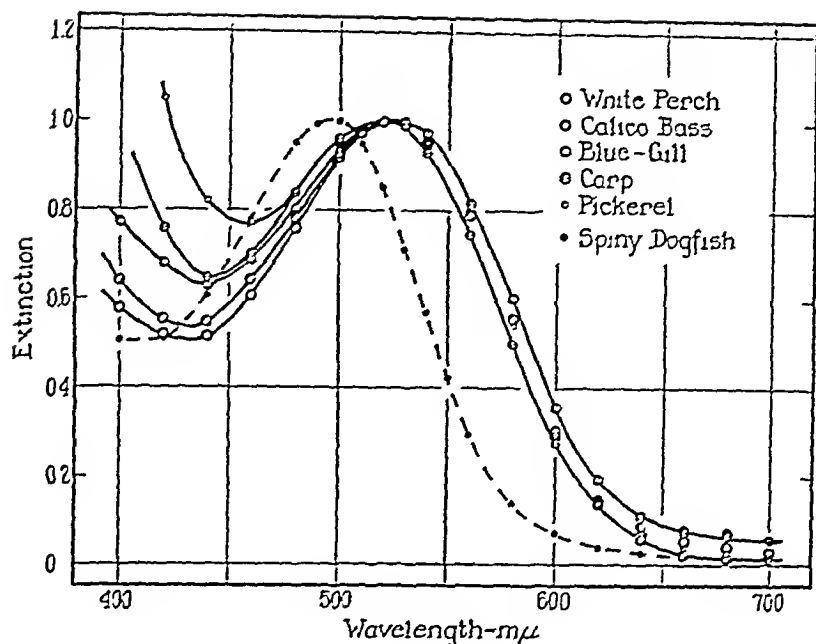


FIG 5 Spectra of porphyropsin preparations from anadromous and fresh-water fishes, and of a typical rhodopsin preparation from the marine spiny dogfish. Digitonin extracts of retinas pre-treated with alum, measured in 1 per cent neutral aqueous digitonin solution. The porphyropsin spectra form a homogeneous group, with maxima at  $522 \pm 2 m\mu$ , the rhodopsin band is narrower, and maximal at about  $500 m\mu$ .

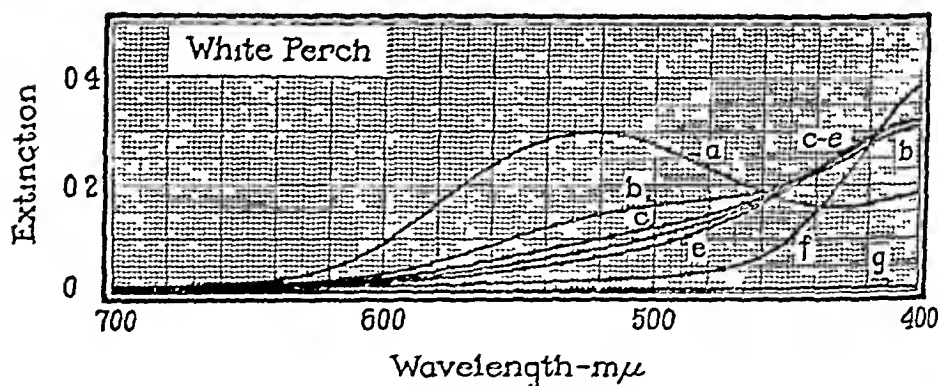


FIG 6 The bleaching of neutral porphyropsin. Extract of eight alum-treated white perch retinas in 2 per cent aqueous digitonin, measured against an equal depth (5 mm) of distilled water. The unbleached solution (a) was exposed to bright light (about 700 foot candles) for 30 seconds. It was then left in darkness and its spectrum re-measured periodically, curve (b) at 1, (c) at 2, (d) at 4, and (e) at 6 minutes from the onset of irradiation. The final yellow product, brought to pH 11 with sodium carbonate, yielded curve (f). Curve (g) is the spectrum of the 2 per cent digitonin alone.

equivalent to true absorption spectra only if porphyropsin bleaches to colorless products. However, as shown below, under all known circumstances porphyropsin bleaches to new pigments, and since at low wavelengths the absorption invariably *rises* on bleaching, difference spectra over this region possess negative values. Had Kottgen and Abelsdorff's measurements extended further into the violet, the confusion of their difference spectra with true absorption spectra could not have occurred. As it was, they observed negative values in the majority of their measurements at 420  $m\mu$ , but dismissed them as "physically meaningless." Actually the difference spectra of porphyropsin like those of rhodopsin, though on occasion useful for descriptive or analytic purposes, are physically meaningless.

On exposure to bright light, porphyropsin solutions bleach very quickly to deep orange, then more slowly to a straw color which, unless exposed to intense irradiation, is maintained indefinitely. I have examined the bleaching processes spectrophotometrically at various pH's, using procedures applied previously to the study of rhodopsin preparations.

Neutral rhodopsin bleaches in a succession of light and "dark" (photic and thermal) processes, of which the latter may account for about half the total fall in extinction at the maximum. I have distinguished three dark components in the bleaching of rhodopsin, only one of which appears regularly in neutral preparations from alum treated retinas, while a second becomes prominent in acidic solutions (Wald, 1937-38, dark processes II and I respectively).

The bleaching of a neutral solution of porphyropsin from white perch is shown in Fig. 6. The spectrum of the unbleached preparation (a) possesses the characteristic maximum at 522  $m\mu$ . The first recorded product of irradiation (b) possesses a broad maximum at about 510  $m\mu$ , and higher absorption than porphyropsin itself below about 460  $m\mu$ . Following irradiation the preparation was left in complete darkness. The absorption continued to fall, maximally in the region of 515-520  $m\mu$ , simultaneously rising below a node at 444  $m\mu$ . This thermal change was virtually complete in about 1 hour (e). Dark reactions account in this instance for 34 per cent of the total fall in extinction at 520  $m\mu$ . The behavior of this preparation is precisely parallel to that of rhodopsin in comparable circumstances (*cf.* Wald, 1937-38, Fig. 3B).

The final product of bleaching (*e*) possesses a spectrum similar to that of retinene<sub>2</sub>. It varies in color reversibly with pH, tending toward orange in acid and pale yellow in alkaline solution. The present residue, brought to pH 11 with sodium carbonate, yielded curve *f*. Such bleached residues yield all their color on extraction with benzine. The benzine extract, transferred to methanol or aqueous digitonin, exhibits a spectrum similar to that of the whole residue, and to retinene<sub>2</sub>. It varies in color with pH as does the whole residue. It apparently owes its spectral properties to retinene<sub>2</sub>.

The light and dark components in the bleaching of porphyropsin differ markedly in their effects. This is most conveniently shown by computation of the difference spectrum which each of these components contributes (Fig. 7). The fall in extinction due to the photic reaction is maximal at about 540 m $\mu$ , and possesses negative values below about 460 m $\mu$ . The difference spectrum of the thermal component is maximal at about 515–520 m $\mu$ , possesses negative values below about 445 m $\mu$ , and a distinct minimum at about 420 m $\mu$ . A similar displacement of maximum distinguishes the photic and thermal components of bleaching in rhodopsin solutions (compare Wald, 1937–38, Fig. 4)<sup>3</sup>.

The sum of the curves shown in Fig. 7 represents the total change in absorption on bleaching neutral porphyropsin. This possesses a maximum at 535–540 m $\mu$ . The difference spectra of fish photopigments reported by Kottgen and Abelsdorff consist simply of the positive portions of this composite function.

In alkaline solution the thermal components of bleaching are greatly accelerated. At pH 9 they are of much the same form as in neutral solution, except that the nodal point below which the absorption rises is displaced to about 430 m $\mu$ . Probably due to further increase in its velocity, no dark component of bleaching has yet been recorded at pH 10.5 or 11. Within 1 minute from the beginning of irradiation at about 25°C the spectrum of the product has reached a stable condition. It is similar to that of the final product of bleaching in neutral solution, brought to the same pH (Fig. 6*f*), and does not change further in darkness (compare Wald, 1937–38, Fig. 7).

<sup>3</sup> It should be noted that in the earlier figure the ordinates of the dark component of bleaching of rhodopsin had been multiplied throughout by 4.

In acid solution the thermal component of bleaching is greatly retarded and probably for this reason a second dark reaction appears. The bleaching of a blue gill preparation at pH 3.65 is shown in Fig. 8. The first recorded product of irradiation possesses a broad hump max

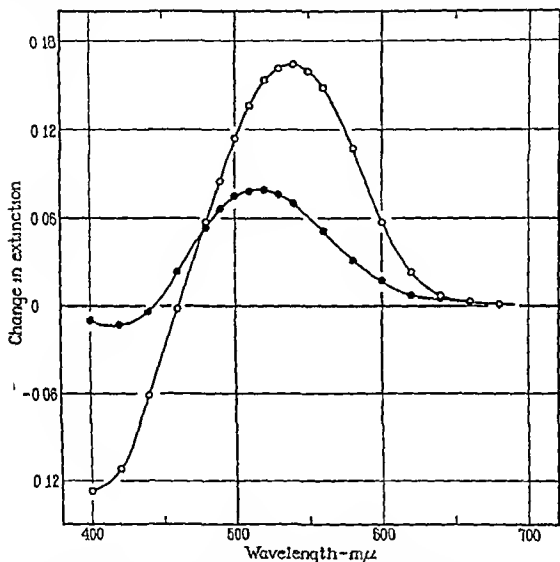


FIG. 7 Changes in absorption which accompany the bleaching of neutral porphyropsin ('difference spectra'), taken from the data of Fig. 6. Positive values indicate decrease and negative values increase in absorption. Open circles show the effect of the light reaction (Fig. 6 *a-b*), closed circles those of the 'dark' processes (Fig. 6 *b-e*). Bleaching due to light is maximal at about 540  $m\mu$ ; that due to thermal reactions at about 515  $m\mu$ .

mal at about 510  $m\mu$  and higher absorption than porphyropsin itself below about 470  $m\mu$  (*b*). Thereafter in darkness the absorption rises slowly in the region of 460  $m\mu$ , simultaneously falling to either side of nodes at about 540 and 425  $m\mu$  (*c, d*). This change is precisely

analogous to dark process I in rhodopsin solutions. Within 10 to 15 minutes this development reaches its peak and the absorption begins to fall over the entire spectrum above about  $410\text{ m}\mu$ . After many hours it reaches a stable condition which is the acidic analogue of the final product in neutral and alkaline solutions (*e*). All these changes are precisely parallel to the behavior of rhodopsin in comparable circumstances (Wald, 1937-38, Figs 5 and 6)

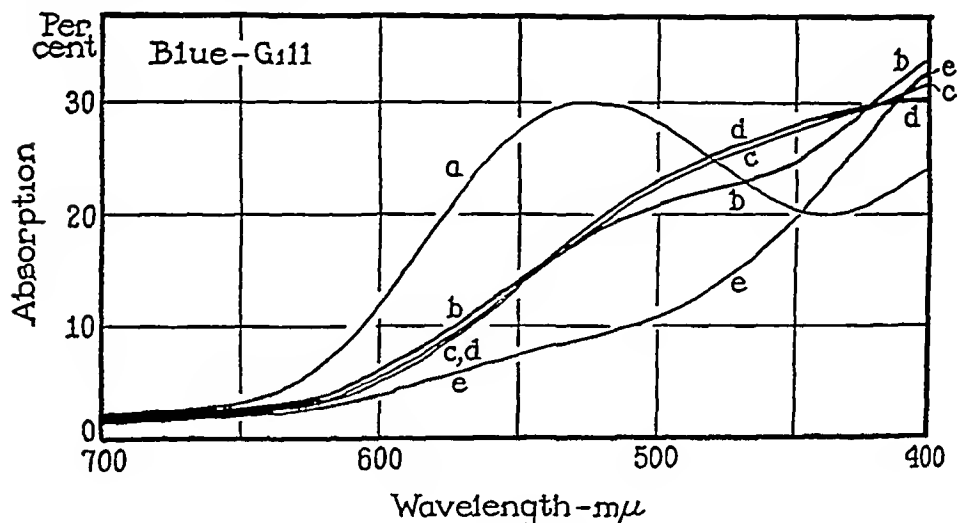


FIG 8 Bleaching of porphyropsin at pH 3.65. The unbleached solution (*a*) was irradiated with about 500 foot candles for 30 seconds, and the spectrum thereafter recorded periodically in darkness, *b* at 2.5, *c* at 5.5, and *d* at 8.5 minutes (retraced at 15.5 minutes).  $26.5^{\circ}\text{C}$ . After 20 hours more in darkness, the spectrum had fallen to *e*.

It may be concluded that in every detail so far examined the behavior of porphyropsin solutions parallels that of rhodopsin, but the spectra of porphyropsin and of all its products of bleaching are displaced uniformly  $20\text{--}30\text{ m}\mu$  toward the red. Porphyropsin in solution bleaches in a sequence of light and dark processes, a final product of which is retinene<sub>2</sub>.

#### *Porphyropsin as Protein*

Many of the observations of Ewald and Kuhne on rhodopsin show it to be protein in nature (Wald, 1935-36). Recently this aspect of rhodopsin chemistry has been further investigated. Hecht and

Pickels (1938) have examined frog rhodopsin in the ultracentrifuge, and conclude from its diffusion and sedimentation properties that it is monodisperse and possesses an apparent molecular weight of 200,000-270,000. Lythgoe and Quilham (1938) have re examined the heat destruction of frog rhodopsin in digitonin solution and find an apparent activation energy of about 45,000 calories per mol. Electrophoretic measurements on rhodopsin solutions from grass and bullfrogs (*Rana pipiens* and *calesbiana*) by the moving boundary method show this pigment to be amphoteric and to possess an isoelectric point in the neighborhood of pH 4.5 (Wald and Rayment, unpublished observations). It may be concluded that rhodopsin is a conjugated carotenoid protein.

The qualitative observations which led to this conclusion have been repeated with calico bass porphyropsin. This pigment does not diffuse through a wet collodion membrane when dialyzed against solutions of phosphate buffer (pH 5.9), digitonin, or egg albumin. It is not precipitated from dilute solution by half saturation with ammonium sulfate, but does precipitate at about 0.8 saturation. Porphyropsin in the retina is destroyed almost instantly by exposure to normal solutions of KOH, HCl, or H<sub>2</sub>SO<sub>4</sub>. In concentrated ammonia water (25 per cent NH<sub>3</sub>) it is decolorized within about 2 minutes, in methanol within 15-30 seconds, in ethanol, chloroform, or acetone within 2 to 3 minutes (19°C). The color of the final product of these treatments in the retina displays the reversible pH lability of retinene<sub>2</sub>; it is deep orange in acid solution, straw colored in neutral, and faint greenish yellow in alkaline media.

Dark adapted retinas retain an intense purple color in concentrated zinc chloride solution, on irradiation they bleach to a bright orange color which is maintained indefinitely. In mercuric chloride solution (pH 3.2) dark adapted retinas turn bright pink, and this color does not change appreciably on irradiation. In mercuric acetate (pH 3.7) retinas turn deep yellow in the dark within 20 minutes at about 20°C, and are not further affected by exposure to light.

Porphyropsin in the retina is destroyed by warming to temperatures above 62°C. At 66 to 67° it is decolorized within 3 to 4 minutes, at 72 to 73° within 30 seconds. The product of heat, like that of chem-

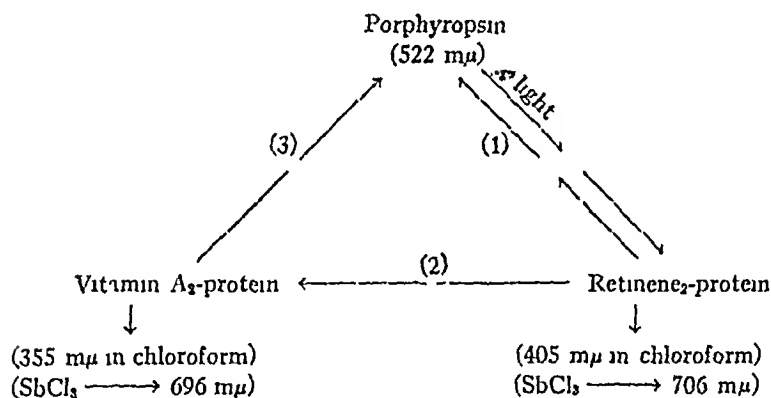


ical destruction, is light straw in color when neutral, and changes in color reversibly with pH

Its non-diffusibility, salting-out properties, and sensitivity to common protein denaturants and to heat indicate that porphyropsin, like rhodopsin, is a conjugated carotenoid-protein. On denaturation, as on irradiation, it yields orange or yellow products and eventually retinene<sub>2</sub>. But the further conversion of retinene<sub>2</sub> to vitamin A<sub>2</sub> characteristic of the "native" retina has not been observed in retinas treated with denaturants. Apparently denaturation, either of the protein residue of porphyropsin itself or of some essential retinal component, perhaps an enzyme, blocks this process.

### CONCLUSIONS

All the foregoing observations may be united in the following diagram of the porphyropsin cycle



Isolation of the retina cuts this cycle at point (3), the processes initiated by light end in the production of a quantity of vitamin A<sub>2</sub>. Denaturation of the retina, or extraction of porphyropsin into aqueous solution virtually eliminates in addition processes (1) and (2), and leaves only the succession of photic and thermal reactions which form retinene<sub>2</sub>. This formulation is not intended to present the actual course of these changes rigorously. Certain of them may be further complicated by intermediate and side reactions. The equations are designed specifically to represent the known empirical relations among the three most stable loci of the porphyropsin system.

In every particular so far examined, in the retina and in solution,

this and the rhodopsin system exhibit parallel behavior. Yet a uniform displacement in spectrum divides all components in each cycle from their analogues in the other. The significance of this relationship is now reasonably clear.

Vitamin A<sub>2</sub> appears to be the next higher homologue of vitamin A, possessing one added ethylenic linkage, and hence one added double bond (Gillam *et al.*, 1938). Hausser, Kuhn, and Smakula (1935) have shown that in a homologous series of synthetic polyenes in benzol solution the addition of a conjugated ethylene group shifts the absorption maximum 20 to 27 m $\mu$  toward the red. The vitamin A<sub>2</sub> maximum in chloroform lies just 27 m $\mu$  from that of vitamin A. This displacement is therefore consistent with the proposed structure.

However, this same range of displacements separates the absorption maxima of all components of the rhodopsin and porphyropsin systems (Fig. 4). The maxima of the retinenes are about 18 m $\mu$ , those of the photopigments about 22 m $\mu$  apart. Similar differences divide the intermediates of bleaching in solution in the two systems.

We may conclude, therefore, that the primary chemical difference between the rhodopsin and porphyropsin systems as a whole is the possession by the latter of one added ethylene group. This accounts not only for all the observed spectral displacements, but explains the otherwise extraordinary parallelism in chemical behavior in the two systems. For the introduction of an added ethylene in the polyene chain should affect the ordinary chemical performance almost negligibly. By this very simple variation of a structure common to all other types of vertebrate, the fresh water fishes have evolved their distinctive visual system.

This situation offers an important commentary on the general problem of color vision. It has been recognized since Helmholtz that trichromatic vision demands three types of retinal elements which vary in spectral sensitivity. In animals like the chicken, pigeon, and certain turtles, whose cones are equipped with three groups of color filters, this requirement can be met with a single cone photopigment (Wald and Zussman, 1938). In other animals including man, where there is no evidence of similar structures, one has been led to assume the existence of three distinct photopigments which vary in their absorption spectra (*cf.* Hecht, 1937). It seems clear from a

wide variety of physiological data that none of these pigments is rhodopsin, and the recent extraction of a distinct cone photopigment, iodopsin, from chicken retinas has confirmed this belief (Wald, 1937 *b*, see also Chase, 1938)

I think it has not previously been stated that in addition to differences in absorption spectra, the hypothetical cone photopigment systems must satisfy a further requirement. Each system must maintain its position relative to the others in all illuminations and at all stages of adaptation. If this condition were not fulfilled, the quality of the sensation would vary widely with adaptation, and the association of a specific color with an illuminant would be impossible. To form a consistent color vision system, therefore, the cone photopigments must exhibit precisely parallel chemical behavior, even to the extent of possessing roughly equivalent kinetics in all their retinal reactions.

Comparison of the rhodopsin and porphyropsin systems shows how both these criteria may be fulfilled simply and elegantly. By small variations in the number or arrangement of double bonds in the polyene nucleus, a number of photopigments might be obtained, varying slightly or widely in their spectral characteristics and consistently parallel in all their chemical activities.

#### SUMMARY

1 In the rods of fresh-water and some anadromous fishes, rhodopsin is replaced by the purple photolabile pigment porphyropsin. This participates in a retinal cycle identical in form with that of rhodopsin, but in which new carotenoids replace retinene and vitamin A.

2 Porphyropsin possesses a broad absorption maximum at  $522 \pm 2 \text{ m}\mu$ , and perhaps a minimum at about  $430 \text{ m}\mu$ . The vitamin A-analogue, vitamin A<sub>2</sub>, possesses a maximum in chloroform at  $355 \text{ m}\mu$  and yields with antimony trichloride a deep blue color due to a band at  $696 \text{ m}\mu$ . The retinene-analogue, retinene<sub>2</sub>, absorbs maximally in chloroform at  $405 \text{ m}\mu$  and possesses an antimony chloride maximum at  $706 \text{ m}\mu$ .

3 Its non-diffusibility through a semi-permeable membrane, salting-out properties, and sensitivity to chemical denaturants and to heat, characterize porphyropsin as a conjugated carotenoid-protein.

4 The porphyropsin cycle may be formulated  $\text{porphyropsin} \xrightleftharpoons[\text{(1)}]{\text{light}}$  retinene: protein  $\xrightarrow{(2)}$  vitamin A<sub>2</sub> protein  $\xrightarrow{(3)}$  porphyropsin Isolation of the retina cuts this cycle at (3), denaturation procedures or extraction of porphyropsin into aqueous solution eliminate in addition (1) and (2)

5 The primary difference between the rhodopsin and porphyropsin systems appears to be the possession by the latter of an added ethylenic group in the polyene chain

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# TEMPERATURE AND CRITICAL ILLUMINATION FOR REACTION TO FLICKERING LIGHT

## IV ANAX NYMPHS

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### I

The first experiments testing the dependence of the form and other properties of the flicker response contour upon temperature<sup>1</sup> failed to disclose any simple relation between critical intensity and temperature. They did demonstrate that for the sunfish *Enneacanthus* and for the nymphs of the dragon fly *Anax* there occurs no appreciable change of the maximum ( $F_{max}$ ) over the range 12.4 to 27.3°, and that the shape of the  $F - \log I_m$  curve is not influenced by the temperature. The general theory of the interpretation of the flicker response contour requires that the first derivative of  $F$  vs  $\log I_m$  with respect to  $\log I$  give a frequency distribution of the elements of effect concerned in the determination of the threshold response (this is *not* a frequency distribution of  $\log I$  thresholds for the receptor or central units providing these effects), at a given  $F$ ,  $I_m$  is the mean intensity required for the activation of a summed total number of the sensory elements sufficient to cause the response to occur. If these elements form at any instant a frequency distribution of thresholds of excitability which is "normal," then the frequency distribution of  $d(1/I)$  should be normal, if these elements fluctuate in their capacity to contribute to the determination of the result measured, then, a finite time being involved in the process of excitation, the distribution of  $d(-\log I)$ , or of  $d(\log I)$  for the effects produced will be normal. The curve of  $F - \log I$  will

<sup>1</sup> Crozier, W J, Wolf, E, and Zerrahn Wolf, G, 1936-37 *a J Gen Physiol* 20, 393 1936-37 *b* 20, 411

<sup>2</sup> 1936-37, *J Gen Physiol* 20, 411 1937-38, 21, 17 313

be a probability integral. In structurally uncomplicated cases<sup>3, 4</sup> this is the fact. In duplex visual systems<sup>5</sup> the separation of the constituent populations of sensory effects is rationally made and tested analytically on this basis. The behavior of the parameters of the probability integral, with respect to number of retinal elements (area),<sup>6</sup> and to light-time fraction in the flash cycle,<sup>7, 8</sup> is entirely consistent with this conception. So also is the qualitative fact that with elevation of temperature the  $F - \log I$  contour is simply moved to a lower place on the temperature scale, without change of shape or change of maximum ordinate.<sup>1, 3</sup> Elevation of temperature simply increases the excitability ( $1/I$ ) of each elementary unit concerned, it cannot be held to modify the magnitude of the contribution made by an element to the determination of the response, since neither the shape nor maximum of the  $F - \log I$  contour is modified. This also supplies a functional proof that the elements in a population behaving in this manner do in fact constitute a homogeneous population: the processes governing excitability are of the same kind in all the elements of such a population, since their arousal is influenced by temperature to quantitatively the same proportionate extent.

It was shown<sup>3</sup> that for the turtle *Pseudemys* the value of  $1/I$  for two fixed levels of  $F$  follows the rule obeyed by the velocities or frequencies of very many biological processes,<sup>9</sup> including among other things such phenomena as the reciprocals of the latent times for photic responses with  $I$  constant.<sup>10</sup> The reciprocals of the critical intensities

<sup>3</sup> 1938 a, *Proc Nat Acad Sc*, 24, 125, 1938 b, 24, 216. 1938-39, *J Gen Physiol*, 22, 311.

<sup>4</sup> 1939-39 a, *J Gen Physiol*, 22, 451, 1938-39 b, 22, 555. 1938, *Proc Nat Acad Sc*, 24, 538.

<sup>5</sup> 1936-37 a, *J Gen Physiol*, 20, 211, 1936-37 b, 20, 393, 1937-38 a, 21, 17, 1937-38 b, 21, 203. 1938, *Proc Nat Acad Sc*, 24, 125, etc.

<sup>6</sup> 1937-38, *J Gen Physiol*, 21, 223. Cf also Crozier, W J, 1937, *Proc Nat Acad Sc*, 23, 71.

<sup>7</sup> 1937-38, *J Gen Physiol*, 21, 313.

<sup>8</sup> 1937-38, *J Gen Physiol*, 21, 463.

<sup>9</sup> Crozier, W J, 1924, *Proc Nat Acad Sc*, 10, 461. 1924-25, *J Gen Physiol*, 7, 189, 1925-26, 9, 531. Crozier, W J, and Stier, T J B, 1924-25 a, *J Gen Physiol*, 7, 429, 1924-25 b, 7, 699.

<sup>10</sup> Hecht, S, 1925-28, *J Gen Physiol*, 8, 291, 1926-27, 10, 781, etc.

for reaction to flicker measure the speeds of the "driving" processes leading to the eventuation of the index response. This is the determination of levels of excitability governing the measured value of the flash illumination responsible for reaction to flicker at a given flash frequency. The Arrhenius equation describes the dependence of  $1/I$  upon temperature,—and with values of  $\mu$ , the temperature characteristic or apparent critical increment, which are found in a number of other biological processes.

When the  $F - \log I_m$  curves for sunfish and *Anax* were examined at three temperatures it was noted<sup>1</sup> that instead of finding  $\log (1/I)$  a rectilinear function of reciprocal absolute temperature, as required by the Arrhenius equation, the plot was concave upward. This could easily result from the complexity of the process measured, if two or more concurrent processes should be simultaneously concerned in determining the sensitivities of the individual elements of excitability. It was accordingly suggested<sup>1</sup> that the phenomena of response to flicker were probably too complex for treatment in a simple, direct way as a function of temperature. The results with *Pseudemys*<sup>2</sup> showed that this conclusion was probably too superficial. It should indeed have been recognized<sup>2</sup> that measurements at three temperatures could not give a clear result in the event that their span includes a critical temperature.<sup>11</sup> On either side of a critical temperature there may obtain a different slope constant ( $\mu$ ) for dependence upon temperature, or a change in velocity without change of  $\mu$ . The former occurs in the data from *Pseudemys*. The peculiar feature of the measurements with the sunfish, an apparent increase of " $\mu$ " with rising temperature, has been resolved by a careful re-examination of this case.<sup>12</sup> It turns out that one of the rare occurrences of a higher  $\mu$  on the higher temperature side of a critical temperature (ca 20°) was responsible for our original deductions,<sup>1</sup> and that in fact  $1/I$  behaves as it should if governed by the velocity of reactions in a catenary chain of which one or the other of two catalytically different steps is in control, depending on the temperature range.

<sup>11</sup> Crozier W J, 1924-25, *J Gen Physiol*, 7, 123, 189. 1925-26 9, 525.  
Crozier W J and Wolf E. 1938-39, *J Gen Physiol* 22, 311.

<sup>12</sup> 1938-39 *J Gen Physiol*, 22, 487.



## II

In the meanwhile a good deal had been learned about the nature of the flicker response contour as obtained with arthropods. Forms such as *Anax*, *Cambarus*,<sup>13</sup> *Uca*, *Apis*, *Drosophila*, provide curves of visual excitability (flicker,<sup>14</sup> visual acuity<sup>14</sup>) which depart in a consistent manner from the probability integral formulation. This has been traced to the fact that these arthropods have large, convex optic surfaces. The departure is consistent with the view that conditions of test which demand the action of a higher intensity of illumination permit the involvement of ommatidia further around the margin of the eye than can be stimulated under conditions of lower illumination. Up to a certain level of intensity, consequently, conditions (such as increase of flash frequency) which necessitate the use of higher intensities for the response, result in a virtual enlargement of the effective retinal area, and thus of the total number of neural elements implicated, and so lead to an augmentation of the level of critical effect (and of  $I$ ) at the point of response.<sup>6</sup> Beyond a certain level of intensity this effect is no longer a factor and the  $F - \log I$  data beyond this point adhere to the typical probability curve. This conception has been tested, with concordant results, through the effects obtained by blocking out parts of the retinal surface in *Anax*,<sup>6</sup> and in *Cambarus*,<sup>13</sup> and by altering the proportion of light time to dark time in the flash cycle.<sup>8</sup> It is also supported by the fact that in an arthropod with the most highly convex eyes (*Cambarus*) the distortion of the  $F - \log I$  curve is more extreme,<sup>13</sup> and particularly by the fact that in a sufficiently flat-eyed form (*Asellus*<sup>4</sup>) no distortion of this kind appears at all.

It is consequently of peculiar interest to re-examine the dependence of the *Anax*  $F - \log I_m$  curve upon temperature with the utmost care and precision possible. The data thus far available<sup>1</sup> indicate unmistakably that the curve of  $\log 1/I$  is probably concave upward. We have considered<sup>1-6</sup> that the shape of the curve could not be said to be significantly altered by temperature, although we have stated the fact<sup>1</sup> that the crude temperature coefficient of  $1/I$  is, as the data stood, to

<sup>13</sup> 1939-40, *J. Gen. Physiol.*, in press

<sup>14</sup> 1937-38, *J. Gen. Physiol.*, 21, 223, 1938-39 a, 22, 451, 1939-40, in press

some extent a function of the flash frequency. The special significance of this case arises in two ways. If the effect referred to be real, we could perhaps obtain a quantitative key to the rôle of intrinsic excitability of the peripheral units in the determination of the form of the  $F - \log I$  contour. In the second place, if it should appear that, apart from this effect, the Arrhenius plot of  $1/I$  vs  $1/T_{ab}$  is unequivocally concave upward, rather than composed of two intersecting straight lines, as in the case of the sunfish,<sup>12</sup> we would be in possession of one of the really rare instances among many biological phenomena thus far examined for which this special condition is to be observed. The theoretical interest of such cases is considerable, their analysis should present no particular difficulty, and is of first rate importance for the theory of temperature characteristics.<sup>9</sup> Experience over a period of some years has demonstrated that our sources of *Anax* larvae provide animals exhibiting a number of features of quantitative consistency with respect to the properties of measurements significant in such an inquiry. The effect sought is in one sense comparatively slight, as could be expected theoretically (see Section IV), the infrequent occurrence of instances of the kind found in the data on *Emmeacanthus*<sup>1</sup> led us to expect that this particular type of dependence on temperature might not, as a matter of sheer probability, occur in *Anax* as well. Certain purely mechanical considerations, already referred to, reinforced this expectation. Our experiments have accordingly been planned in a manner calculated to provide a critical test of the curvilinear or rectilinear character of the data to be obtained with *Anax* when displayed upon an Arrhenius grid.

### III

At closely spaced intervals of temperature, with a homogeneous<sup>13</sup> group of individuals, measurements were made of critical intensities for response of *Anax* nymphs to visual flicker at each of two flash frequencies,  $F = 20$  and  $F = 55$ . The first flash frequency is at the center of the region of the  $F - \log I_m$  curve which departs from the probability integral curve<sup>14</sup> the second is in the upper, orthodox part of the curve. The time order of the temperatures used was arranged

<sup>13</sup> Crozier, W J, 1936, *Proc Nat Acad Sc*, 22, 412. Crozier, W J, and Holway, A H 1937, *Proc Nat Acad Sc* 23, 23, etc.

to reveal drifts of excitability with time and experimental history during the observations, none were noted. The uniform technic of observation and of calculation of the entries in the tables has been discussed in some detail in earlier papers,<sup>1</sup> and need not be repeated.

Table I contains observations drawn from our data secured with different lots of *Anax* larvae over a period of several years, showing the kind of reproducibility

TABLE I

Mean critical flash intensities, and the P E of the dispersions, from observations on marginal response to visual flicker in different lots of ten individuals of *Anax junius* nymphs, over a period of 4 years, at 21.5°, with light time fraction in the flash cycle = 50 per cent. Data from earlier reports<sup>14</sup> and (boldface type) from the present experiment.

<i>F</i> = 20/sec	
log <i>I<sub>m</sub></i>	log P E 1/ <i>I<sub>1</sub></i>
<i>ml</i>	<i>ml</i>
$\bar{2}$ 4881	$\bar{3}$ 2248
$\bar{2}$ 4850	$\bar{4}$ 9845
$\bar{2}$ 4876	$\bar{4}$ 7237
$\bar{2}$ 4729	$\bar{4}$ 7236
$\bar{2}$ 4778	$\bar{4}$ 8494
<i>F</i> = 30/sec	
$\bar{2}$ 7403	$\bar{4}$ 9216
$\bar{2}$ 7449	$\bar{3}$ 4360
$\bar{2}$ 7486	$\bar{3}$ 1225
$\bar{2}$ 7416	$\bar{4}$ 8608
$\bar{2}$ 7497	$\bar{4}$ 8976
<i>F</i> = 55/sec	
$\bar{1}$ 7356	$\bar{2}$ 4538
$\bar{1}$ 7071	$\bar{3}$ 8599

obtained. There is, of course, no reason at all for expecting identity of critical intensities, or of intrinsic variability, in lots of individuals from different sources. The sort of difference shown by the data in Table I is also found in experience with other kinds of animals<sup>12, 16</sup>. At *F* = 20 the new observations (*I<sub>m</sub>*) are consistently just a little below those obtained in 1935-36 (at the same season

<sup>16</sup> 1938-39, *J. Gen. Physiol.*, 22, 463

of the year), and also at  $F = 55$ . This is of no importance for the subsequent analysis.

Table II contains values of mean critical intensities at respectively  $F = 20$  and  $F = 55$ , at various temperatures. The temperatures

TABLE II

Mean critical intensities  $I_m$ , and the probable errors of the dispersions ( $P E_{I_1}$ ) at various temperatures for two flash frequencies ( $F = 20$  per second, and  $F = 55$  per second), for nymphs of *Anax junius*.  $t_{corr}$  is the mean temperature during the period of test (see text). Equally long light interval and dark interval in the flash cycle. The same ten individuals were used throughout. Three observations on each of these gave thirty determinations of  $I_1$  at each point.

$t_{corr}$	$F_{20}$		$F_{55}$	
	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$
8 52	2̄ 8366	3̄ 1339	0 0426	2̄ 1804
10 6	2̄ 7953	3̄ 0730	1̄ 9821	2̄ 3021
12 6	2̄ 7194	3̄ 0565	1̄ 9736	2̄ 3197
13 7	2̄ 7062	4̄ 9411	1̄ 9186	2̄ 0370
15 15	2̄ 6933	3̄ 1464	1̄ 8467	2̄ 1123
16 7	2̄ 6401	4̄ 9661	1̄ 8411	2̄ 1602
18 05	2̄ 6887	4̄ 9690	1̄ 7924	2̄ 1136
19 8	2̄ 6421	4̄ 9598	1̄ 7334	3̄ 9901
21 5	2̄ 4876	4̄ 7237	1̄ 7071	3̄ 8599
	2̄ 4729	4̄ 7236		
	2̄ 4778	4̄ 8494		
23 42	2̄ 4214	4̄ 7993	1̄ 6341	3̄ 8386
25 40	2̄ 3619	4̄ 7766	1̄ 6540	3̄ 8150
27 15	2̄ 2801	4̄ 6436	1̄ 4790	3̄ 7401
28 85	2̄ 2082	4̄ 7574	1̄ 4086	3̄ 6183
30 45	2̄ 1670	4̄ 5109	1̄ 3647	3̄ 6002
33 40	2̄ 0633	4̄ 3764	1̄ 2294	3̄ 4697
35 8	3̄ 9547	4̄ 1507	1̄ 0730	3̄ 7817
			1̄ 0580	3̄ 1021
			1̄ 1129	3̄ 5813

listed are mean temperatures in the aquaria during the period of observation.<sup>1</sup> The animals were dark adapted for several hours at a particular temperature, in a thermostat. The temperature of the water in the individual aquaria changes by several tenths of a degree at most during the observations, depending on the distance from room

temperature (*ca* 21.5°) These deviations, adjusted to the mean interval of observation by thermometer readings, are probably less in the substance of the *Anax* nymphs Experience with these animals, however, unmistakably indicates<sup>17</sup> that the rate of adjustment of body temperature under the conditions imposed is so rapid that the effective temperature cannot be in any case far from that tabulated Deviations of mean temperature of 0.2° could not possibly affect the direction of the interpretation which the data demand The same ten individuals were used throughout the experiment The behavior of these individuals, as objectively indicated by the variations of the measurements, was very similar

## IV

We have first to examine the plottings of  $\log 1/I_m$  as a function of  $1/T_{obs}$ , with reference to rectilinearity These are given in Fig 1 It is apparent that, as found previously,<sup>1</sup> the temperature coefficient for  $1/I$  does increase with rising temperature The increase is smooth and regular Thermodynamically this is impossible unless the net result (end-point effect) is determined by the summation of the effects of two or more concurrent processes, each proceeding independently over the whole range of temperature Close inspection of the data shows, making all reasonable allowance for the variation of temperature at each point and the (constant)<sup>18</sup> relative variability of  $1/I$  for each  $I$ , that the course of the measurements in Fig 1 cannot be described other than by a curve In the somewhat (but only superficially) similar sunfish data<sup>12</sup> the use of a curve rather than two separate lines is forbidden by the obvious properties of the data The situation in the sunfish<sup>12</sup> and turtle<sup>3</sup> requires the assumption that at a critical temperature, 20° ± in the former case, 30° in the latter, there occurs a change from one pacemaker process to another, with the sunfish the controlling process above 20° has a higher  $\mu$  than that below, the "break" in the curve at the critical temperature is clean and definite The mechanism of such changes presents a problem requiring separate discussion<sup>2</sup>

<sup>17</sup> Crozier, W J, and Stier, T J B, 1924-25, *J Gen Physiol*, 7, 429

<sup>18</sup> Crozier, W J, 1935-36, *J Gen Physiol*, 19, 503, Crozier, W J, Wolf, E, and Zerrahn-Wolf, G, 1937-38, *J Gen Physiol*, 20, 363, 393, 21, 223, 463

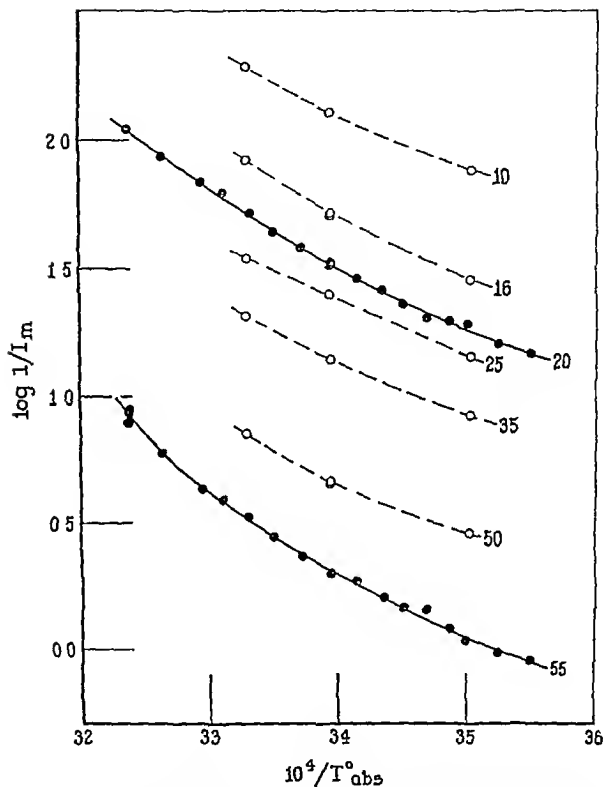


FIG. 1  $1/I_m$  for response of *Anax* nymphs to visual flicker at various flash frequencies ( $t_L = t_D$ ) as a function of temperature. Solid dots: data of Table II. Open circles: data from an earlier report<sup>1</sup>.  $\log 1/I_m$  is not a rectilinear function of  $1/T$ ; the curve is continuously concave upward.

It is a striking fact in the large body of data having to do with temperature and the speeds or frequencies of biological processes that extremely few instances occur in which the temperature characteristic  $\mu$  increases smoothly with rise of temperature, giving a continuously curved Arrhenius plot concave upward. Such a graph indicates plainly that at least two different processes are contributing simultaneously and independently to the governance of the index end-point. That such curves are found to be extremely rare is a powerful argument in support of the general proposition that rectilinearity in the Arrhenius plot properly signifies essential simplicity in the pacemaking reaction,<sup>19</sup> this is supplemented by the fact that the constancy of the relative *variation* of performance requires the operation of a single rather than of a compound system of control.<sup>20</sup> When the Arrhenius plot for over-all velocities in a purely chemical system is concave upward the method for analysis of the data is, however, perfectly straightforward<sup>21</sup> and can be used to resolve the complexity of the situation with, in favorable cases, recognition of the contributing factors.

In the present data we have additional features providing internal confirmation of the applicability of the analysis.

# V

The experiment was designed to reveal the occurrence of any change in the form of the  $F - \log I$  curve produced by altering the temperature. One flash frequency ( $F = 20$ ) was chosen near the center of that portion of the *Aa* curve which departs widely from the probability integral<sup>22</sup> where the departure (in terms of  $F$ ) is greatest. The other flash frequency ( $F = 55$ ) was chosen on the part of the curve

<sup>19</sup> Crozier, W. J., 1924-25, *J. Gen. Physiol.*, 7, 189.

<sup>20</sup> Crozier, W. J., and Federighi, H., 1924-25, *J. Gen. Physiol.*, 7, 565. *Proc. Nat. Acad. Sc.*, 11, 80. Crozier, W. J., and Stier, T. J. B., 1924-25, *J. Gen. Physiol.*, 7, 429, etc. Crozier, W. J., 1929, in Murchison, C., *The foundations of experimental psychology*, Worcester, Clark University Press, pp. 45-127. 1935, *Déterminisme et variabilité*, Paris, Hermann et Cie, 56 pp.

<sup>21</sup> Norrish, R. G. W., and Rideal, E. K., 1923, *J. Chem. Soc.*, 123, 696, 1689, 3202. Hinshelwood, C. N., 1929, *The kinetics of chemical change in gaseous systems*, Oxford, Clarendon Press, 2nd edition, 266 pp.

<sup>22</sup> *J. Gen. Physiol.*, 1936-37, 20, 363, 393. 1937-38, 21, 223, 463.

which adheres to the probability integral. If  $F_{max}$  is really unaffected by change of temperature, and if the shape of the curve is unmodified, then the difference between  $\log I_m$  for  $F = 20$  and  $F = 55$  should be constant at all temperatures. This, of course, means *statistically* constant, since each  $I_m$  carries with it a measure of the dispersion of the distribution of  $I_1$ 's from which it is obtained. In the lower portion of the  $F - \log I$  graph the departure from the fundamental probability integral is easily modified, in *Anax*, by altering the light time fraction<sup>5</sup> or the retinal area.<sup>6</sup> The theory of the departure from the fundamental curve<sup>18, 11, 4</sup> is that in the lower part of the graph increasing critical intensities involve slightly larger effective retinal areas, because of the convexity of the optic surface. The same result could, however, conceivably be brought about by increasing the photic excitability of the individual receptor units. It would not be surprising to find that increase of temperature should do this, so that the discrepant part of the curve should be made to approach the probability integral more closely. On the other hand, increase of temperature lowers the critical illumination at any fixed  $F$ , with retinal area constant, so that in the distorted part of the graph the net result might easily turn out to be a counterbalancing of the increasing sensitivity of the retinulae by the mechanically reduced efficiency of the critical illumination. A careful consideration of the variation of  $I_1$  at  $F = 20$  and at  $F = 55$  should make it possible to detect the action of two such factors. If  $F_{m, z}$  is found to be independent of temperature<sup>1</sup> as it is in other instances not involving the matter of gross optic morphology<sup>2, 12</sup> it cannot be maintained that the total number of available sensory elements is affected.

The differences between  $\log I_m$  for  $F = 20$  and  $F = 55$  (Table II) are plotted in Fig. 2. It is apparent that with the possible exception of the measurements at  $35.8^\circ$  the differences are in no sense significant. With this exception, the fluctuation in  $\Delta \log I_m$  is noticeably greater at  $15-16^\circ\text{C}$  than elsewhere, a fact not without possible meaning in view of the subsequent analytical discussion. Slight day-to-day differences in excitability do not influence these comparisons, inasmuch as the measurements at  $F = 20$  and  $F = 55$  at each temperature were made on the same day. The observations in one set at  $F = 55$  for  $35.8^\circ$  required the use of critical intensities at a comparatively un-



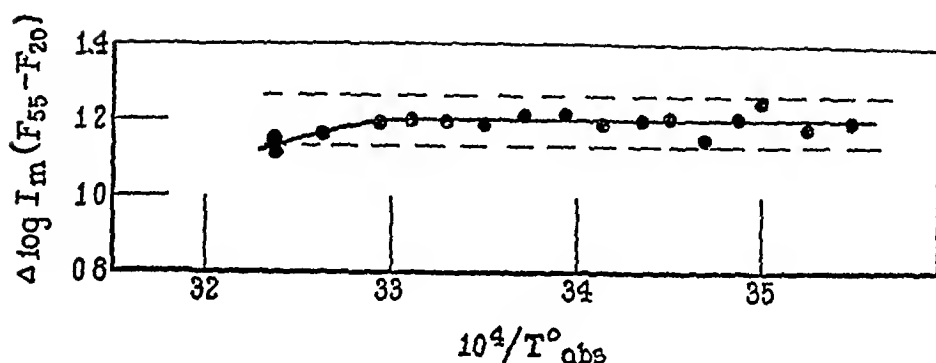


FIG 2 As a test of the constancy of the shape of the  $F - \log I_m$  curve with temperature varied, the interval  $\Delta \log I_m$  between  $\log I_m$  for  $F = 20$  and  $F = 50$  is plotted as a function of temperature. The dashed lines are drawn at  $\pm 2 \times$  mean  $P E_{\Delta}$ . Only at  $35.8^\circ$  is there suggestion of change. See text.

TABLE III

Mean critical intensities for response of *Anax* at  $35.8^\circ$ , at several flash frequencies, and mean critical flash frequencies, and mean critical flash frequencies at higher intensities,  $t_L = t_D$ .

$F/\text{sec}$	$\log I_m$	$\log P E_{I_1}$	$\log I$	$F_m$	$P E_{I_1}$
30	$\bar{2} 1569$	$\bar{4} 4017$	$\bar{1} 00$	$54.38$	$0.435$
40	$\bar{2} 3816$	$\bar{4} 7126$			
45	$\bar{2} 6143$	$\bar{4} 7575$			
50	$\bar{2} 7517$	$\bar{3} 0318$			
55	$\bar{1} 0580$	$\bar{3} 1021$			
60	$\bar{1} 0730$	$\bar{3} 7817$	$0.00$	$60.59$	$0.408$
	$\bar{1} 1129$	$\bar{3} 5813$		$61.29$	$0.377$
	$\bar{1} 9581$	$\bar{3} 1523$		$61.64$	$0.220$
				$61.35$	$0.299$
				$61.33$	$0.257$
				$61.58$	$0.316$

fortunate steep place on our calibration charts<sup>23</sup> of intensities in the apparatus, that this did not fundamentally influence either  $I_m$  or

$\sigma I_1$  is, however, made clear by the other tests made at the same  $F$  with other lamps and their more favorable calibration curves (Tables II and III). It is indicated by the subsequent discussion that in all probability the departure of  $\Delta \log I_m$  at  $35.8^\circ$  is due to the exceptional behavior of  $\log I_m$  at  $F = 55$ , rather than at  $F = 20$ ,  $\log I_m$  at  $F = 55$  is 0.03 to 0.09 log unit lower than it "ought" to be, and  $I_1$  is too variable.

The theory of the asymmetrical  $F - \log I$  function for arthropods with quite convex eyes holds that, up to a certain intensity increase of  $I$  is able to recruit activity from the periphery of the optic surface because of light leakage through the substance of the eye.<sup>22, 14</sup> At a particular flash frequency, therefore, such as  $F = 55$  if the required critical intensity is reduced—as by raising the temperature—it should be possible to bring the intensity to a level such that the involvement of tangentially affected receptor units would be a significant factor. At temperatures up to  $33^\circ$  there is no evidence that this is actually the case (Fig. 2). Examination shows that a temperature of  $35-36^\circ$ , however, brings  $\log I_m$  just to the upper edge of the lower portion of the  $F - \log I_m$  graph which at  $21.5^\circ$  departs from the probability integral,<sup>14</sup> so that additional recruitment of marginal elements might clearly be detectable. Since, however, determinations at other flash frequencies demonstrate a change in the form of the curve at  $35.8^\circ$ , it is necessary to appeal also to recruitment based upon the increase of peripheral excitabilities with rise of temperature. These data are contained in Table III. By comparison with determinations at  $21.5^\circ$ <sup>14</sup> it is seen that the differences  $\Delta \log I_m$  between determinations at  $21.5^\circ$  and at  $35.8^\circ$  increase slightly but rather regularly around  $F = 55$ .

$F$	$\Delta \log I_m$ ( $\log I_m$ at $21.5^\circ$ ) - ( $\log I_m$ at $35.8^\circ$ )
20	0.525
30	0.586
40	0.592
45	0.574
50	0.589
55	0.655
60	0.610

It would not be unreasonable to find that with decreasing flash time ( $1/F$ ) this effect could be decreased. As Table III shows at  $\log I = 1.50$  (corresponding<sup>14</sup> to  $\log I = 2.1+$  at  $21.5^\circ$ )  $F_m$  is 61.33 to 61.64 (mean = 61.475). This agrees very well with the corresponding 61.42 at 1.84 obtained at  $21.5^\circ$ . The theoretic value of  $F_{ma}$  was there taken as = 61.48 for the data at  $35.8^\circ$  the same value is best for the older measurements, at  $21.5^\circ$ ,  $F_{max}$  for the  $I_m$  data.

was 61.1, and in the present series 60.9. The agreement must be regarded as quantitative, on a probability grid (Fig. 3) the slopes are identical, showing that  $\sigma_{\log I}$  for the underlying frequency distribution of sensory effects is not materially affected by temperature.  $P E_{1F_1}$  (Table III) is of the order of magnitude found in earlier measurements,<sup>18</sup> taking into account the fact that the curve is shifted on the  $\log I$  axis, but the values at  $F = 60-61$  are a little high.

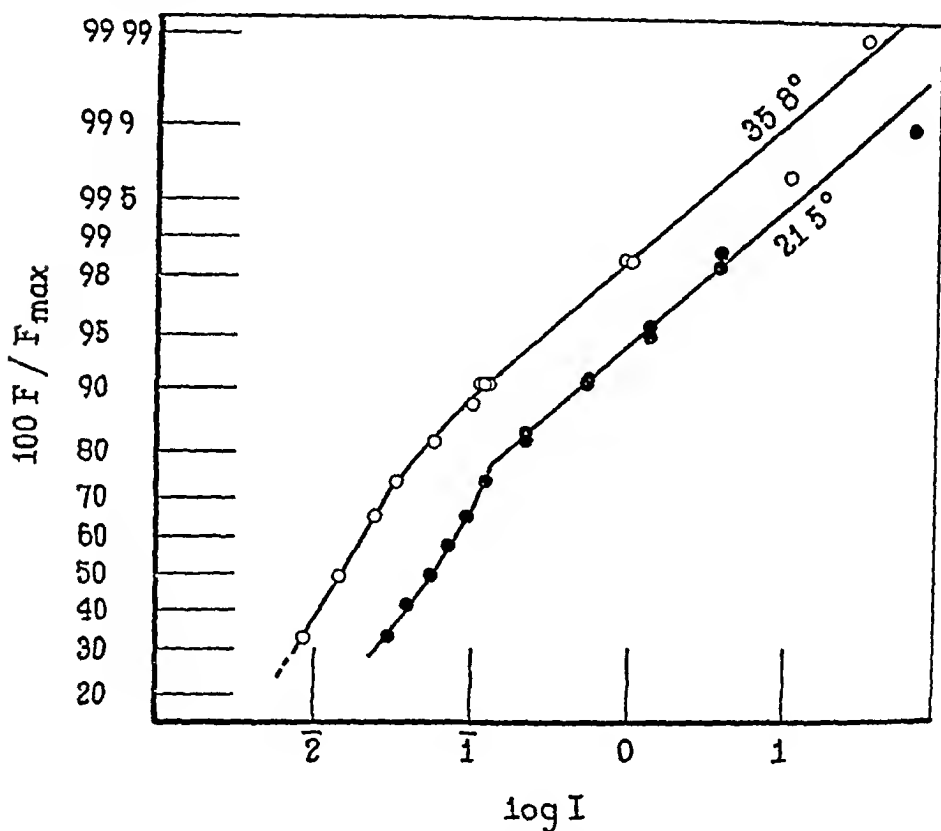


FIG. 3.  $F$  vs  $\log I_m$  on a probability grid, at  $35.8^\circ$  and at  $21.5^\circ$ . Discussed in the text. (The point of largest departure, at  $\log I = 1.0$ , deviates by only 0.2 per cent of 61.5.)

The lower part of the  $35.8^\circ$  graph, the discrepant portion, is, however, a little closer to that for  $21.5^\circ$  than the upper, straight part. The junction of the two segments of the graph (Fig. 3) is definitely more rounded, and runs to a higher  $F$ , at  $35.8^\circ$  than at  $21.5^\circ$ . This plainly indicates that in this part of the graph the lowering of critical intensity at fixed  $F$  induced by raising the temperature has also brought with it a slight reduction in the effective retinal area. There is no sign that increased peripheral excitabilities have sufficed to really counterbalance

this effect. We are justified, however, in supposing that such a factor is operative to some extent. The evidence is mainly indirect. At constant temperature the variation of critical intensity and the mean critical intensity are in direct proportion.<sup>20</sup> This is the relationship expected if  $1/I$  is a measure of the driving force required to eventuate the response.<sup>1, 2, 26</sup> Similarly, if a velocity of (chemical) change is a measure of the driving potential, we find the variation of physiological effects proportional to such a velocity to be directly proportional to the mean velocity. Clearly, if the variation is intrinsic to the reacting or performing living system concerned, the magnitude of the proportionality constant should bear some relationship to the complexity of the controlling events. Examples in which the variation of performance is still proportional to the measure of average performance, but with a different proportionality constant, have been encountered in studies of the relation between temperature and frequency or speed of activity,<sup>24</sup> so that this general contention is not without factual support. Analogous instances are given by the geotropic orienting performance of young rats.<sup>25</sup> In the present data we are concerned to discover if, with temperature as independent variable, the relation between  $I_m$  and the variation of  $I_1$  exhibits any differences at  $F = 20$  and  $F = 55$ . We have indicated reasons for supposing in advance that at  $F = 20$  the variation in performance should be greater. This should be reflected in a wider variance of the flash intensity required to enforce the index behavior employed as an end point. The greater variance is expected because, as increasing temperature reduces the required critical flash intensity ( $F$  being fixed) it also reduces the chance of photic involvement of ommatidia around the periphery of the optic surface. At the same time it must be presumed that elevation of temperature will increase the excitability of these peripheral elements. So that we consider two opposing influences to be at work in the determination of the critical effect requiring motor response to the moving flashes. It does not require the assumption that this critical effect will be of the same magnitude or any energy scale to deduce that the variance of the critical intensity will be enhanced as a consequence. The standard by which this enhancement is to be gauged is provided by the observations at  $F = 55$ . For temperature above 33 the critical illumination falls above that for which (at 21.5°) the recruitment of marginally excitable ommatidia has a significant influence on the form of the curve.<sup>22</sup> Hence, by comparison with the state of affairs at any constant temperature, the variation data for  $F = 20$  must necessarily be expected to fall above those at  $F = 55$ . Fig. 4 demonstrates that this is indeed the fact. Except for  $I_m$  at  $t = 35.8^\circ$  which for reasons already discussed shows excessive variation, the measurements at  $F = 20$  exhibit a higher variation at all temperatures than do those at  $F = 55$ . With *Anax* larvae it has been found<sup>26</sup> that the relative sensitivities of individuals in a lot of ten tend to be maintained for several hours. With certain other forms e.g. various teleosts<sup>1, 12, 16</sup> this is not the case. The effect is obliterated in *Anax* when parts of the eyes are covered.<sup>2</sup> The reasons for this

<sup>24</sup> Crozier, W J, and Stuer T J B, 1926-27, *J Gen Physiol*, 10, 185

have been discussed<sup>22</sup> It should be expected that at higher temperatures a larger proportion of the variance of critical intensity might be found to be "within individuals" as compared with "between individuals," despite the lowering of the critical intensity Rise of temperature should accentuate the individual fluctuations of excitability, and decrease the chance of persisting individual

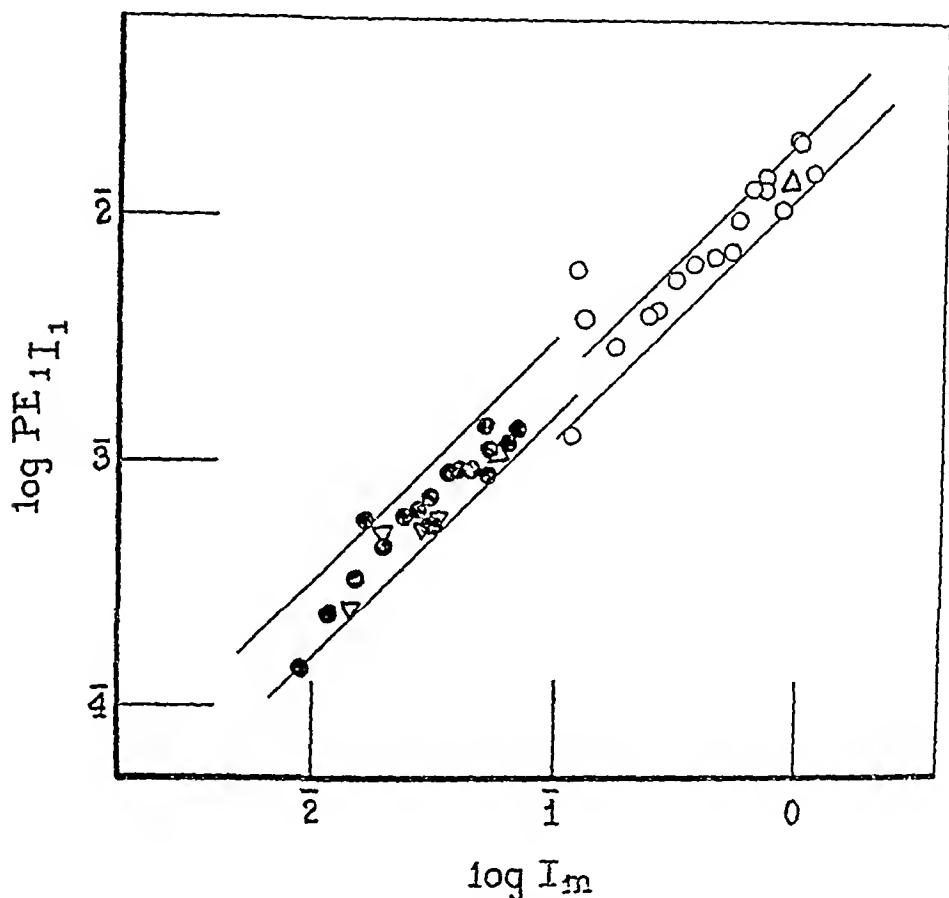


FIG. 4 The variation of  $I_1$  at various temperatures (Table II), for two flash frequencies, solid dots,  $F = 20$ , open circlelets,  $F = 55$ . Triangles give data from Table III. At  $F = 55$ ,  $t = 35.8^\circ$ , the fluctuation of  $I_1$  is exceptional, otherwise, below  $F = 55$  the variation is consistently higher than at  $F = 55$  or above, see text.

differences. Examination shows that at  $35.8^\circ$  the mean rank order numbers for sensitivity are not retained in successive sets of readings, whereas there is distinct correlation at  $21.5^\circ$ . On the other hand the proportion of the total variance due to the differences between individuals, in a set of readings at one  $F$ , is just as great as at lower temperatures.

It is to be clearly understood that the differences we have been discussing in this section are in one sense small. Their internal consistency in a group of very carefully controlled observations nevertheless shows objectively that a definite realistic dignity must be granted to them. Their analytic interpretation is an obligation which cannot be successfully avoided. They have an inescapable significance for the evaluation of any interpretive theory of the mechanism responsible for the observations. In the great majority of biological investigations this obligation is ignored.

Question may very properly be raised as to the rôle of intensity vs quantity of light in a flash with reference to the implication of marginally excitable ommatidia. The facts show that when the flash time is reduced, with cycle time ( $\tau_c$ , frequency) constant, the critical intensity declines.<sup>5</sup> From the data obtained by change of the light time fraction,<sup>8</sup> with  $F$  constant, one can compute for each flash in intensity the flash time corresponding to the temperature which requires the same intensity, at  $F = 20$  this flash time of course decreases as temperature rises (except at 33–36°, the decline is almost rectilinear). However, without correction for the changing value of  $F_{max}$ ,<sup>3</sup>  $\log I_m$  declines more rapidly with decrease of  $t_L$  at  $F = 55$  than at  $F = 20$ . Hence it must predominantly be a matter of *intensity* rather than of quantity of light (either in a flash, or as an average during the comparatively prolonged interval required for observation) which determines the involvement of marginally situated ommatidia. This is of course, quite consistent with the fact that Talbot's law has nothing to say as to the basis for response to flicker ( $\tau_c$ , at constant flash intensity  $F$  *increases* as the light time fraction is *reduced*).<sup>7, 8</sup> The important point is that these considerations support the view that penetration of the tangential margins of the eye, in these arthropods, which is a function of intensity primarily, is the basis for the recruitment of additions to effective receptor area, this cannot be determined by mere modifications of peripheral excitability. The latter have, however, a subsidiary influence, as demonstrated by the second order effects at  $F = 20$ . The failure of  $F_{ma}$  to be in any way affected by temperature, over the range 8–36°,

<sup>25</sup> *J. Gen. Physiol.* 1937–38, 21, 313, 463

shows that the total number of available sensory elements, if at all influenced by temperature, must show a negligible temperature coefficient. It is only within the zone of intensities for which the recruitment effect is perceptible that the effect is detectable at all. The special effect of comparatively very high temperature at  $F = 55$  is traced to the fact that  $I_m$  is then brought within the zone of intensities for which the rôle of peripheral excitabilities is significant. The effect of exposure to  $36^\circ$  is quickly and freely reversible, and therefore is not due to "injury."

The analysis of the dependence of  $I_m$  upon temperature may then proceed with some confidence on the basis that  $I_m$  at  $F = 55$  is (save above  $33^\circ$ ) uninfluenced by the effect of temperature upon peripheral excitability. The data further demonstrate (Section VI) that at  $F = 20$  the influence of increased temperature in decreasing the critical intensity must rather exactly balance the opposite effects due to the decreased marginal penetrating action of the lowered intensity. The constant relative variation of  $I_1$  over the range of temperatures, with  $F = 20$  (Fig 4), is itself a kind of proof of this.

## VI

The plots in Fig 1 show that  $\log 1/I_m$  is related to  $1/T^\circ_{ab}$ , by a curve continuously concave upward. Writers who have been disposed to disapprove of the attempt to measure temperature characteristics of biological processes by means of the Arrhenius equation [velocity  $\propto \exp(-\mu/RT)$ ] have quite without exception failed to realize the significance of such cases, and the unusual rarity of their occurrence.<sup>26</sup> Their importance was referred to in an early survey of biological temperature functions.<sup>27</sup> They indicate unequivocally that more than one process contributes simultaneously to the determination of the observed end-point, their rarity unmistakably implies that for the great majority of temperature-controllable organic processes simplicity of the governing mechanism is self-evident, this is entirely

<sup>26</sup> Cf. Discussion appended to paper by Hoagland, H., 1936, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 4, 267.

Burton, A. C., 1936, *J. Cell and Comp. Physiol.*, 9, 1.

Korr, I. M., 1937, *J. Cell and Comp. Physiol.*, 10, 461.

<sup>27</sup> Crozier, W. J., 1924-25, *J. Gen. Physiol.*, 7, 189 (see p. 192).

supported by the properties of variation of performance in these processes. In chemical systems this is a commonplace, and the type of procedure required for further elucidation has been known for a long time<sup>26</sup>. It is not without significance that the only definite biological instances of this sort previously known have involved the performance of systems in which it is quite obvious that at least two processes are concerned. This was indicated long ago<sup>27</sup> for speed of parthenogenetic activation of echinoderm eggs by acid, a more elaborate demonstration is provided by Korr's data on respiration of sea urchin eggs<sup>28</sup>. The straightforward analysis of these data<sup>29</sup> shows without question that, among other things, two different respiratory processes are here normally proceeding simultaneously. It is not to be understood that cases fail to arise in which two (or more) processes individually influenced in different ways by temperature may not provide an Arrhenius graph *con ex* upward, a model instance of this type was carefully examined long since<sup>31</sup>. The point is that a value of  $\mu$  smoothly and continuously increasing with rise of temperature signifies the concurrent influence of two (or more) processes with different values of  $\mu$  over the whole of the investigated temperature range.

Ideally one requires the possibility of experimentally isolating and in some way directly identifying the proposed contributory processes. This is not difficult in some cases<sup>29, 30</sup>. Even in the case of "simple" physicochemical reactions this is, of course, not always feasible, "wall reactions" complicating gas reactions provide a clearly analyzable type<sup>28</sup>. For a situation such as the present one analysis can justifiably be made in a formal manner, with two ends in view: to provide a rational account of the data, and to supply a guide for subsequent tests. The rationality of the analysis is attested by internal properties of the data. The specific hypotheses resulting can be rather easily put to proof.

The analysis involves one basic consideration which is probably

<sup>26</sup> Cf<sup>21</sup> and Taylor, H. S., 1931. A treatise on physical chemistry, chapter XV, New York, D. van Nostrand Co. Bauer, W. H. and Daniels, F., 1934, *J. Am. Chem. Soc.*, 56, 2014.

<sup>28</sup> Korr, I. M., 1937, *J. Cell and Comp. Physiol.* 10, 451.

<sup>29</sup> To be given in another place.

<sup>31</sup> Crozier, W. I. and Stier, T. J. B., 1925-26 *J. Gen. Physiol.* 9, 49.



strange, or at least unfamiliar. The discussion of curves of visual functions which are (in fact or ideally) symmetrical with  $\log I$  as abscissa has customarily regarded the first derivative of the curve with respect to  $\log I$  as a frequency distribution of excitabilities<sup>32</sup>. This is quite incorrect. The derivative is a frequency distribution of *effects* produced as a consequence of the basic excitabilities<sup>33</sup>. The fact that a statistical basis must be granted for these effects<sup>34</sup> leads directly to the conclusion that the curve of total effect *vs*  $\log I$  must be a normal probability integral, regardless of the form of the frequency distribution of the instantaneous excitabilities of the individual contributing units (so long as their number is large). The proper measure of excitability is  $1/I$ , not  $1/\log I$ . Consequently it is with  $1/I$  that we must deal in considering the application of the equation for "energy of activation". This is the basis for testing the form of  $1/I$  on an Arrhenius grid, by plotting  $\log 1/I$  against  $1/T_{abs}^{\circ}$  (Fig 3).

A formal resolution of a curvilinear graph in these coordinates, of course, cannot be *proved* to give a *unique* interpretation, even within the limits of statistical propriety. The method is one of trial. The physical limitations of a biological system force one to work within certain thermal limits. The slopes toward the ends of the curve (Fig 3) provide suggestions as to the orders of magnitude of the two main contributory  $\mu$ 's. This does not require that there be only two such, of course it does not necessarily follow that the curve of the data can be constructed from two such processes alone. The construction of the curve of the data requires the selection of suitable  $\mu$ 's and also the selection of suitable relative positions on the  $\log K$  axis for their graphs. While this at first sight may seem to provide large latitude in the choice of  $\mu$ 's, in fact it does not, with well determined values of  $K$  (i.e., of  $1/I$ ) the requirements of the curve are rigorous.

This process has been applied to the curves in Fig 3. The result of many trials is given in Fig 5. The points at  $35.8^{\circ}$  for  $I = 55$  have been discounted, for reasons already discussed rather fully (Section V). With this exception the curves for  $I = 20$  and  $I = 55$  are sensibly

<sup>32</sup> Hecht, S., 1924-25, *J Gen Physiol*, 7, 235, 1927-28, 11, 255. Hecht, S., and Wolf, E., 1928-29, *J Gen Physiol*, 12, 727.

<sup>33</sup> Cf. Crozier, W. J., 1936, *Proc Nat Acad Sc*, 22, 412, etc.

<sup>34</sup> See <sup>33</sup> <sup>34</sup> and Crozier, W. J., 1937, *Proc Nat Acad Sc*, 23, 71.

identical in form. Two processes with respectively  $\mu = 19,200$  and  $\mu = 3,500$ , and with velocities equal at  $ca\ 15.9^\circ$ , give a faithful account of the data. It has not been possible to find two others which do

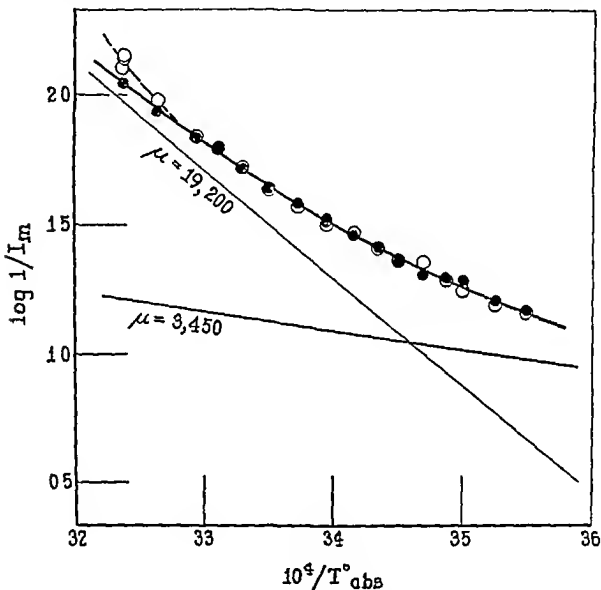


FIG. 5. The measurements at  $F = 20$  and at  $F = 55$  are brought together on the  $\log 1/I_m$  axis (cf. Fig. 1) by vertical displacement (data of  $F = 55 \times \text{antilog } 1.205$ ). The form of the two curves is the same (the exceptional points at  $F = 55, t = 35.8^\circ$  are discussed in the text). The curve shows continuous increase of slope as the temperature rises and thus of the temperature characteristic  $\mu$ . This signifies the concurrent participation of at least two independent processes with different  $\mu$ 's determining the excitability ( $1/I$ ) for response to flicker. The curvature found is accurately reproduced by the summation of the two processes whose velocity curves are shown below, one with  $\mu = 19,200$  the other with  $\mu = 3,400$ . Their velocities are equal at  $15.9^\circ$ .

It is to be remembered that the "velocities" are respectively to be measured by the proportionate contributions of the two processes to the determination of the end-point result, and they may well contribute in different ways

In one subsidiary feature the curves in Fig 5 are additionally suggestive. It has been pointed out that near a temperature at which there cross the curves for two opposed or parallel independent processes contributing to the governance of a particular result one must

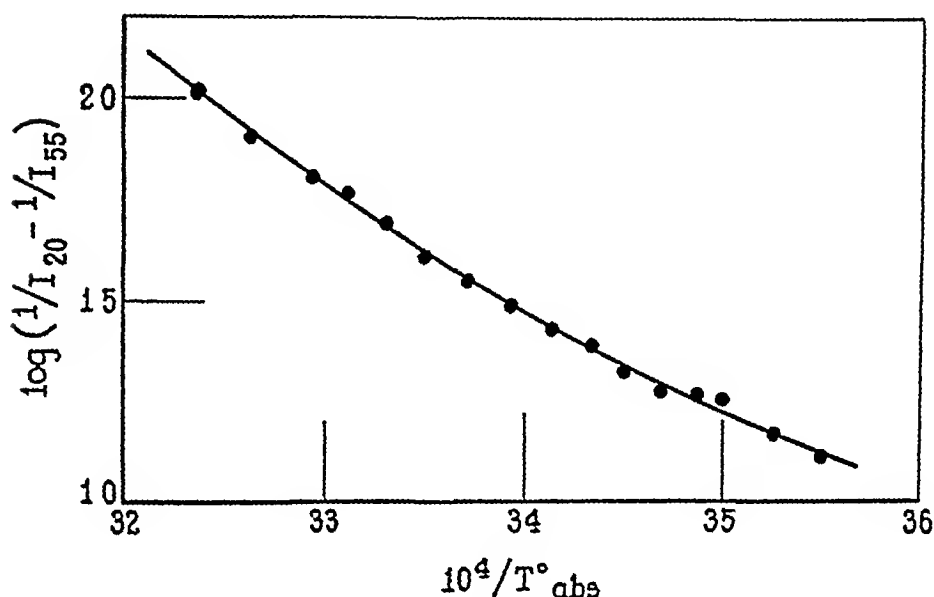


FIG 6 The extra excitation required to pass from the level of response at  $F = 20$  to that for  $F = 55$  is measured by  $(1/I_{20} - 1/I_{55})$ . This quantity also gives a means of estimating  $\mu$  for the processes governing excitability. The curve drawn is that already shown in Fig 5

expect excessive variation of performance<sup>35</sup>. It is probably not an accident that at 15–16° (Fig 5) the scatter of mean  $1/I_m$  is greater than elsewhere. (This has of course nothing to do with the relation between  $I_m$  and  $\sigma I_1$ .)

Finally, confirmation of part of the basis for this type of analysis is independently obtainable from the data. The point involved is that if  $1/I$  is the proper measure of excitability for a particular magnitude

<sup>35</sup> Crozier, W. J., and Federighi, H., 1924–25, *J. Gen. Physiol.*, 7, 151

of sensory effect (*i.e.*, of  $F$ ,<sup>36</sup> in this case), then, granted no change in the form of the  $F - \log I$  curve, the difference between  $1/I_m$  for  $F = 55$  and for  $F = 20$  must follow the same curve as a function of temperature as does  $1/I_m$  for any given level of  $F$ . The argument is that the sensory excitation necessary to produce the constant increase of effect represented by the difference between reaction at  $F = 20$  and at  $F = 55$  will require a driving potential which is less the higher the temperature, and is measured by  $1/I$ . Thus whereas  $\Delta \log I_m$  is constant (Fig. 2), the intensity difference required to produce this constant difference in sensory effect declines exponentially with rise of temperature—and in the present data the curve must have the same form as in Fig. 5. Fig. 6 shows that this requirement is satisfied, this of course follows if the  $\log 1/I$  curves for  $F = 20$  and  $F = 55$  have the same shape as a function of  $T^\circ$ , so that  $I_{55}$  and  $I_{20}$  are in constant ratio.

## VII

A limiting process with  $\mu$  as low as 3,400 (Figs. 5 and 6) could be essentially one of hydrodiffusion, or it could represent the outcome of opposing processes in equilibrium. No certain deductions can be made with respect to it. In the present case it cannot be supposed that we have to do with a balance between the decreasing retinal area of operation of the critical flash intensity, on the one hand, and in the opposite direction the elevation of peripheral excitabilities brought about by rise of temperature, because (below  $33^\circ$ ) above  $F = 55$  the  $F - \log I$  curve does not rise.

With respect to  $\mu = 19,200$ , however (Figs. 5 and 6) more definite notions may be entertained. A variety of evidence clearly suggests<sup>30</sup> that it is associated with dehydrogenations (*i.e.*, fundamentally, with activation of  $H^+$ ), and probably in a number of instances with the first steps in the burning of simple sugars.<sup>37</sup> This may not be difficult to test, by way of suitably designed metabolic experiments. Thus by modification of the glycogen or other carbohydrate reserves, or of catalysts for dehydrogenation, the limiting rôle of the process for which  $\mu = 19,200$  might be obliterated. In this manner clues might

<sup>36</sup> 1937-38 *J. Gen. Physiol.*, **21**, 17.

<sup>37</sup> Cf. <sup>30</sup> and Gould, B. S., and Sizer, I. W., 1938, *J. Biol. Chem.*, **124**, 269; Crozier, W. J., 1924-25, *J. Gen. Physiol.*, **7**, 189.

be available as to the nature of the events governing the capacity to react to flickering light. The temperature characteristics found<sup>17</sup> for frequency of breathing movements in *Anax* nymphs (11,500 and 16,200) are quite different. It does not seem profitable to regard the chemical control of this visual excitability as located in the peripheral receptors, since in that event the enhancement of the "dark" process by elevation of temperature would be expected to have the effect of enlarging the population of elements of effect, just as reduction of the light time fraction does, this is not found.

#### SUMMARY

At fixed flash frequency ( $F = 20$ ,  $F = 55$ ) and with constant light time fraction (50 per cent) in the flash cycle, the critical illumination  $I$  for response of *Anax* nymphs to visual flicker falls continuously as the temperature rises. The temperature characteristic  $\mu$  for the measure of excitability ( $1/I$ ) increases continuously with elevation of temperature. The form of the  $F - \log I$  curve does not change except at quite high temperature ( $35.8^\circ$ ), and then only slightly (near  $F = 55$ ),  $F_{max}$  is not altered. The very unusual form of the  $1/I$  curve as a function of temperature is quantitatively accounted for if two processes, with respectively  $\mu = 19,200$  and  $\mu = 3,400$ , contribute independently and simultaneously to the control of the speed of the reaction governing the excitability, the velocities of these two processes are equal at  $15.9^\circ$ .

We wish to express our thanks to Mrs. E. Wolf for her assistance in the experiments.

## BIOELECTRIC POTENTIALS IN VALONIA

### II EFFECTS OF ARTIFICIAL SEA WATERS CONTAINING LiCl, CsCl, RbCl, OR NH<sub>4</sub>Cl

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Measurements of  $P D$  across the protoplasm of cells of *Valonia macrophysa*, Kütz, have furnished valuable information<sup>1</sup> as to the behavior of the protoplasm toward Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> ions. It has accordingly seemed desirable to extend this study to ions of similar chemical properties, not present in significant amounts in ordinary sea water. The present report gives the results of a few exploratory measurements<sup>2</sup> using various dilutions of modified sea waters in which LiCl, CsCl, RbCl, or NH<sub>4</sub>Cl was substituted for NaCl and KCl.

In the experiments with various dilutions of natural and KCl rich sea waters,<sup>1</sup>  $P D$  time curves of two different types have been observed.

When natural sea water is replaced by undiluted KCl rich sea water the  $P D$  rises<sup>3</sup> rapidly to a maximum, falls to a minimum, and then rises more slowly to a second maximum. This characteristic fluctuation of the  $P D$  (found also with *Valonia* sap and with pure 0.6 molar KCl) has been attributed<sup>4</sup> to changes caused

<sup>1</sup> (a) Damon E B and Osterhout W J V *J Gen Physiol*, 1929-30 13, 445 (b) Damon E B *J Gen Physiol*, 1932-33 16, 375 (c) Damon, E B *J Gen Physiol* 1937-38, 21, 383

<sup>2</sup> The experiments reported here were performed at Bermuda in 1930. It was originally planned to make a detailed study of the effects of each of these salts but this program has actually been carried out only in the case of KCl.<sup>1b</sup> Since this work has been interrupted and cannot be continued by the writer it seems worth while to publish these results in spite of their fragmentary nature.

<sup>3</sup> In the experiments reported in this paper, the  $P D$  across the protoplasm is in all cases directed inward in the sense that positive current tends to flow from the external solution through the protoplasm into the vacuolar sap.

<sup>4</sup> (a) Damon E B *J Gen Physiol* 1929-30 13, 207 (b) Damon, E B, *J Gen Physiol* 1931-32 15, 525

by the penetration of  $K^+$  into the aqueous middle layer<sup>5</sup> of the protoplasm. Although the later changes in  $P D$  are complex, it is found that the initial rise in  $P D$  with KCl-rich sea waters is reproducible, and varies in a regular manner with the concentration of potassium,  $C_K$ , in the external solution according to the equation

$$P D = 59.1 \log (BC_K + D) \quad (\text{mv at } 25^\circ \text{ C})$$

where  $B$  and  $D$  are constants. It is assumed that the first maximum in the  $P D$ -time curve occurs before the concentrations in the inner layers of the protoplasm have been affected by the penetration of  $K^+$ , in other words, that only the external surface layer of the protoplasm (the  $X$  layer) is concerned in the initial rise in  $P D$ .

Similar  $P D$ -time curves are observed with small dilutions of KCl-rich sea waters, the initial rise in  $P D$  decreasing as the dilution is increased. With these solutions, also,  $K^+$  plays an important part in determining the  $P D$ .

With diluted natural sea water and with high dilutions of KCl-rich sea waters, however, the  $P D$ -time curves have a different shape. When these solutions are substituted for ordinary sea water, the  $P D$  rises rapidly to a definite value at which it remains approximately constant for some time, the  $P D$  increases with increasing dilution. The  $P D$  is independent of small changes in the concentration of  $K^+$  in these solutions. The greater is the concentration of  $K^+$  in the undiluted sea water, the higher is the critical dilution at which  $K^+$  ceases to influence the  $P D$ . In sea waters containing both KCl and NaCl, the concentration effect above the critical dilution is determined solely by the activity of NaCl in the external solution.

From these results, certain inferences have been drawn in regard to the diffusion of  $K^+$ ,  $Na^+$ , and  $Cl^-$  in the outer surface layer of *Valonia* protoplasm (the  $X$  layer).

The concentration effect with these sea waters above the critical dilution has been interpreted as a diffusion potential in the  $X$  layer of the protoplasm, involving only the  $Na^+$  and  $Cl^-$  ions which are coming out from the vacuole. (The outward diffusion of  $K^+$  from the vacuole is evidently prevented by the mechanism responsible for the accumulation of KCl in the cell sap.) The relative mobilities of  $Na^+$  and  $Cl^-$  in the  $X$  layer may then be calculated from this diffusion potential with the help of the familiar Nernst equation. If the mobility of the  $Cl^-$  ion is arbitrarily taken as unity, the mobility of  $Na^+$  is found<sup>1c</sup> to be 0.11.

The electrical potential gradient in the  $X$  layer produced by the outward diffusion of  $Na^+$  and  $Cl^-$  tends to oppose the inward diffusion of other cations from the external solution. This explains the failure of  $K^+$  to enter the protoplasm and influence the  $P D$  when the dilution is greater than the critical value. While it has proved possible to calculate the critical dilutions for KCl-rich sea waters, this calculation is based on certain assumptions which cannot be applied without

<sup>5</sup> For the theory of protoplasmic layers, see Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, 11, 83, *Biol. Rev.*, 1931, 6, 369, *Ergebn. Physiol.*, 1933, 35, 1013.

modification to other cations than  $K^+$  (or to other organisms than *Valonia*). Nevertheless, similar phenomena are to be expected with *Valonia* when other cations are substituted for  $Na^+$  and  $K^+$  in diluted sea water

The apparent relative mobility of  $K^+$  in the outer or *X* layer of the protoplasm has been calculated from the initial rise in *P D* in undiluted  $KCl$  rich sea waters, assuming that this change in *P D* arises from a new diffusion potential in the *X* layer. If the mobility of  $Cl^-$  is taken as unity, the apparent mobility of  $K^+$  (recalculated to agree with the new value of 0.11 for  $Na^+$ ) is found to be 18. In this calculation, the additional assumption was made that the partition coefficients of  $KCl$  and  $NaCl$  between water and the *X* layer are equal. Since it is not improbable that the partition coefficient for  $KCl$  is actually considerably higher than that for  $NaCl$ , the value, 18, may be too high. The sign and magnitude of the concentration effect with small dilutions of  $KCl$  rich sea waters, however, indicate that the mobility of  $K^+$  must be several times greater than that of  $Cl^-$ .

We may now consider some similar experiments using sea waters containing  $Li^+$ ,  $Cs^+$ ,  $Rb^+$ , or  $NH_4^+$

The composition<sup>6</sup> of the modified sea waters used in these experiments is given in the following table, where the symbol *M* is used to represent  $Li$ , or  $Cs$ , or  $Rb$  or  $NH_4$

<i>M</i>	0.500 molar	$Cl$	0.570 molar
$Ca$	0.011 ‘	$Br$	0.001 ‘
$Mg$	0.054 ‘	$SO_4$	0.028 “
		$HCO_3$	0.003 ‘

The pH of each solution was adjusted to the same value as that of ordinary sea water as shown by the color of thymol blue. These stock solutions are designated as *LiCl sea water*, etc. In some experiments these stock solutions were diluted with natural sea water; the resulting solutions are described by such expressions as *0.1 molar RbCl in sea water*. For studying the concentration effect, the stock solutions were diluted with a solution of glycerol 8.7 per cent by weight in distilled water. (This solution is approximately isotonic with Bermuda sea water.) Such a diluted solution may be called, for example, a *d fold dilution of  $NH_4Cl$  sea water*. Here the dilution, *d*, represents the number of liters of diluted solution containing 1 liter of the stock solution.

<sup>6</sup> Based on a formula for artificial sea water recommended by McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Publication No. 251*, 1917.



The apparatus and experimental methods have been described in earlier publications<sup>4</sup> To avoid waste in experiments with solutions containing RbCl and CsCl, the same technic was employed as in the measurements with natural *Valonia* sap<sup>4b</sup>

## I

*Modified Sea Water Containing LiCl*

In its influence on the P D with *Valonia*, the  $\text{Li}^+$  ion appears hardly distinguishable from the  $\text{Na}^+$  ion, at least so far as the outer surface layer of the protoplasm (the X layer<sup>5</sup>) is concerned In eleven meas-

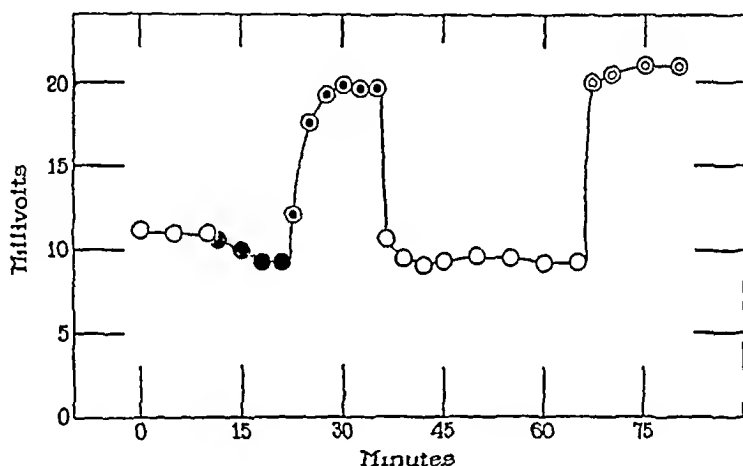


FIG 1 P D-time curve showing the change in P D across *Valonia* protoplasm when ordinary sea water (open circles) is replaced by LiCl-sea water (shaded circles) and comparing the concentration effects with a twofold dilution of LiCl-sea water (double circles, shaded centers) and with an equal dilution of natural sea water (double circles, open centers) LiCl-sea water contains 0.5 M LiCl

urements with LiCl-sea water, the P D-time curves resembled the curves with potassium-free NaCl-sea water the P D generally fell to a value less than the value in ordinary sea water, but greater than zero Such a curve is shown in Fig 1 A representative P D-time curve with potassium-free NaCl-sea water has been presented in an earlier paper<sup>7</sup>

With both the NaCl-sea water and the LiCl-sea water, however, the behavior is liable to be erratic, these potassium-free solutions are

<sup>7</sup> Damon, E B, *J Gen Physiol*, 1932-33, 16, 378 (curve marked  $C_K = 0$ )

evidently somewhat injurious and prone to cause secondary changes in the protoplasm. A  $\text{LiCl}$  sea water containing the same concentration of  $\text{K}^+$  as ordinary sea water would probably give better results. More prolonged measurements, which should be possible with such solutions, might reveal effects at inner layers of the protoplasm where  $\text{Li}^+$  may behave differently from  $\text{Na}^+$ .

With diluted  $\text{LiCl}$  sea water, the  $\text{P D}$  time curves are similar to the curves with diluted natural sea water, the concentration effect with  $\text{LiCl}$  sea water, however, is somewhat smaller. Fig 1 compares the concentration effects with twofold dilutions of  $\text{LiCl}$  sea water and of natural sea water. Four such measurements gave values for the concentration effect with a twofold dilution ranging from 6 to 10 mv, average, 8 mv. One measurement with a fivefold dilution gave 22 mv. The values for two- and fivefold dilutions of natural sea water, reported in an earlier paper,<sup>14</sup> were 11.4 and 25.2 mv. From comparison of these concentration effects, we may conclude that the mobility of  $\text{Li}^+$  in the  $X$  layer of *Valonia* is perhaps slightly greater than that of  $\text{Na}^+$ .

## II

### *Modified Sea Water Containing CsCl*

The  $\text{Cs}^+$  ion, like the  $\text{Li}^+$  ion, resembles the  $\text{Na}^+$  ion in its effect on the  $\text{P D}$  with *Valonia*. Two measurements with  $\text{CsCl}$  sea water gave  $\text{P D}$  time curves like the curves with potassium free  $\text{NaCl}$  sea water. One of these curves is shown in Fig 2.

Three measurements of concentration effect with a fivefold dilution of  $\text{CsCl}$  sea water showed  $\text{P D}$ 's somewhat higher than the  $\text{P D}$  with an equal dilution of ordinary sea water, this is illustrated in Fig 2. The same result was obtained whether the diluted  $\text{CsCl}$  sea water was applied after the diluted natural sea water, or before it as shown in the figure. This larger concentration effect might seem to indicate that the mobility of the  $\text{Cs}^+$  ion in the  $X$  layer is less than that of the  $\text{Na}^+$  ion.

It is not certain, however, that with the fivefold dilution the  $\text{Cs}^+$  ion really plays any significant part in the  $\text{P D}$ . It may be that this dilution is greater than the critical dilution for  $\text{CsCl}$  sea water, and that only  $\text{Na}^+$  and  $\text{Cl}^-$  ions diffusing out from the vacuole are actually

concerned in the P.D. The P.D. to be expected in such a case may be estimated by extrapolating the straight line which represents the P.D.-dilution curve of KCl-sea water<sup>8</sup> above its critical dilution. The extrapolated value for a fivefold dilution is in good agreement with that observed with the diluted CsCl-sea water

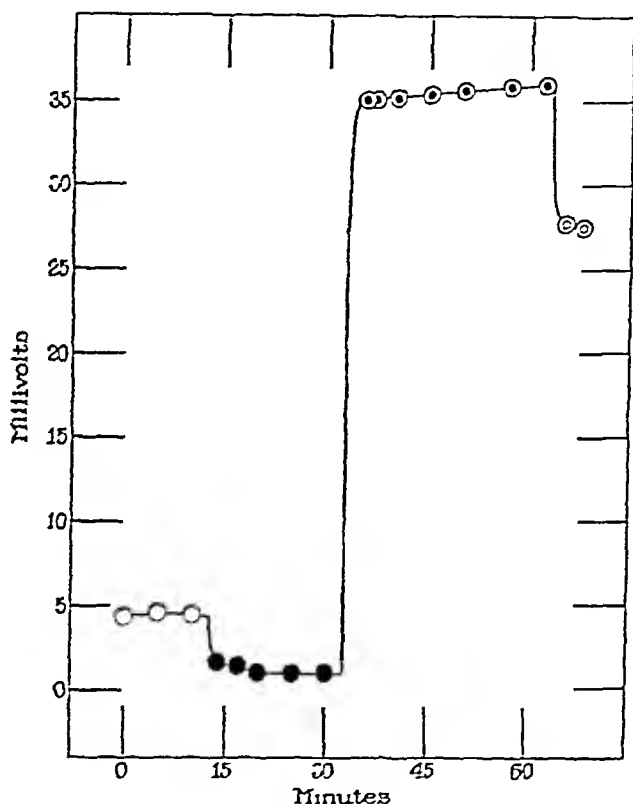


FIG. 2. P.D.-time curve showing the change in P.D. across *Valonia* protoplasm when ordinary sea water (open circles) is replaced by CsCl-sea water (shaded circles) and comparing the concentration effects with a fivefold dilution of CsCl-sea water (double circles, shaded centers) and with an equal dilution of natural sea water (double circles, open centers). CsCl-sea water contains 0.5 M CsCl.

Accordingly, while we may conclude from the data with undiluted CsCl-sea water that the mobility of  $\text{Cs}^+$  in the X layer is not very different from that of  $\text{Na}^+$ , these measurements of concentration effect do not show conclusively which of the two mobilities is the higher.

<sup>8</sup> Damon, E. B., *J. Gen. Physiol.*, 1937-38, 21, 388, Fig. 2, curve A.

## III

*Modified Sea Waters Containing RbCl*

Sea waters containing RhCl, like KCl rich sea waters, increase the inwardly directed  $P D$  across the protoplasm of *Valonia*. The curves showing the variation of  $P D$  with time are more or less similar to the curves with KCl rich solutions. With solutions containing RbCl, however, the second rise in  $P D$  (ascribed to changes at inner layers of the protoplasm) follows so soon after the initial change that the time curve shows only a point of inflection instead of a well defined maximum and minimum.  $P D$  time curves of this type are found occasionally with KCl rich solutions,<sup>9</sup> in such cases, the value of the  $P D$  at the point of inflection is about the same as the value at the first maximum in curves of the usual KCl type.

Curve A of Fig. 3 shows a  $P D$  time curve of this sort observed with RbCl sea water. The initial change in  $P D$  (to the point of inflection) was about 53 mv. In another measurement with RbCl sea water, the time curve had a well defined maximum and minimum, but here the initial rise was only 33 mv.

Three measurements with 0.1 molar RbCl in sea water gave  $P D$  - time curves in which the initial rise was indicated, at the best, by an ill defined point of inflection. The best of these curves is shown as curve B in Fig. 3. In this curve, there is apparently an inflection point at about 21 mv above the  $P D$  with ordinary sea water. In these experiments the  $P D$  finally reached surprisingly high values; in one case, the  $P D$  after 1 hour was 60 mv greater than the value with ordinary sea water.

In two measurements with 0.05 molar RhCl in sea water the changes in  $P D$  were small and gradual. When these solutions were replaced by 0.1 molar RhCl in sea water, however, the  $P D$  rose rapidly and passed through a well defined maximum. This behavior will be discussed later.

The data furnished by the two measurements reported in curves A and B of Fig. 3 are probably the most reliable values which we have for calculating the apparent relative mobility of  $Rb^+$  in the  $\lambda$  layer of

<sup>9</sup> Damon E. B., *J. Gen. Physiol.*, 1929-30 13, 215 1932-33, 16, 384

*Valonia* This calculation is based on the same assumptions as were used in an earlier paper<sup>1b</sup> in calculating the apparent relative mobility of  $K^+$ . It is supposed that the initial change in P.D. (to the point of inflection) represents a diffusion potential set up in the  $X$  layer of the protoplasm. It is further assumed that the coefficients for the dis-

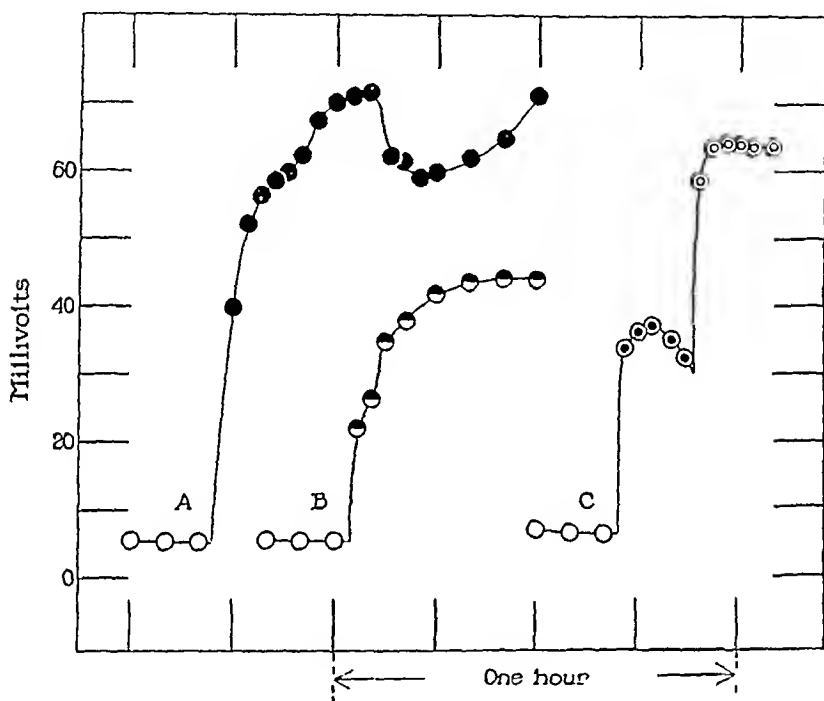


FIG. 3 Time curves showing changes in P.D. across *Valonia* protoplasm when ordinary sea water (open circles) is replaced by modified sea waters containing RbCl. In curve A the external solution is RbCl-sea water (shaded circles), in curve B it is 0.1 molar RbCl in sea water (half-shaded circles). Curve C illustrates the concentration effect with diluted RbCl-sea water: fivefold dilution, double circles, shaded centers; 25-fold dilution, double circles, open centers. RbCl-sea water contains 0.5 M RbCl.

tribution of NaCl, KCl, and RbCl between sea water and the  $X$  layer all have the same value. Using the values which have already been determined for the apparent relative mobilities of  $Na^+$ ,  $K^+$ , and  $Cl^-$ , the relative mobility of  $Rb^+$  may then be computed with the help of the equation (derived from the familiar Henderson formula) which was used in computing the relative mobility of  $K^+$ . From the

measurement with RbCl sea water (curve A) it is found that the mobility of  $\text{Rb}^+$  is 11 times as great as that of  $\text{Cl}^-$ , from the measurement with 0.1 molar RbCl in sea water (curve B), the value is found to be 10.

Since the mobility of  $\text{Rb}^+$  in the X layer is much greater than that of  $\text{Cl}^-$ , and since the behavior of  $\text{Rb}^+$  is similar to that of  $\text{K}^+$ , it is to be expected that the concentration effect with RbCl sea water will resemble the concentration effect with KCl sea water. That is, we may expect that the initial change in  $P D$  produced by diluted RbCl sea water (if the dilution is not too great) will be less than the corresponding change produced by the undiluted solution. We may expect further that as the dilution is increased a critical value will be reached, above which  $\text{Rb}^+$  plays no part in the  $P D$ , and the  $P D$  increases with increasing dilution. The  $P D$  dilution curve will therefore pass through a minimum.

These predictions are confirmed by measurements with fivefold and 25 fold dilutions of RbCl sea water. Thus, three measurements with the fivefold dilution showed an initial rise in  $P D$  of 31 to 33 mv, considerably less than the value, 53 mv, found in the measurement with the undiluted solution shown in Fig 3, curve A. In five measurements with the 25 fold dilution, the change in  $P D$  was greater than with the fivefold dilution, the values varying from 36 to 60 mv, average, 50 mv. The  $P D$  time curve marked C in Fig 3 shows the results of an experiment with these two diluted RbCl sea waters.

#### IV

#### *Modified Sea Waters Containing $\text{NH}_4\text{Cl}$*

The behavior of sea waters containing  $\text{NH}_4\text{Cl}$  proved to be very similar to that of the corresponding sea waters with RbCl, except that the changes in  $P D$  were even larger than those produced by either RbCl or KCl.

One experiment with  $\text{NH}_4\text{Cl}$  sea water showed extremely large fluctuations in  $P D$ , somewhat like those characteristic of KCl rich solutions, but much more rapid. It was obvious that reliable values for the first maximum in such a  $P D$  time curve could be obtained only with a measuring instrument of much shorter period than the

Compton electrometer used in these measurements. To avoid this difficulty, the present investigation was limited to solutions in which the concentrations of  $\text{NH}_4\text{Cl}$  were considerably smaller than 0.5 molar.

Nine measurements were carried out using 0.1 molar  $\text{NH}_4\text{Cl}$  in sea water. The best P D -time curve obtained in this series is shown as curve A in Fig. 4. The P D's observed with these solutions were very high; in this instance, 87 mv greater than the P D in ordinary sea water. As in the experiments with  $\text{RbCl}$ , the initial change in P D was marked, not by a maximum in the P D -time curve, but by a point of inflection, the position of which was often indefinite. In the curve shown in Fig. 4, the initial rise was about 68 mv. In three other curves where the position of the inflection point could be determined approximately, the rise in P D to this point varied between 20 and 53 mv, the average of all four values was 44 mv.

When the sea water containing  $\text{NH}_4\text{Cl}$  was replaced by ordinary sea water, the P D -time curve (as shown in Fig. 4) closely resembled the curves obtained when  $\text{KCl}$ -rich solutions were followed by ordinary sea water.<sup>4a</sup> Precisely the same behavior was observed in experiments with  $\text{RbCl}$ . The changes in P D (rapid fall to a minimum, rise to a maximum, followed by a slow fall) are just the reverse of the characteristic changes observed when  $\text{K}^+$  enters the protoplasm from  $\text{KCl}$ -rich sea waters. It may accordingly be assumed that these changes in ordinary sea water are produced by  $\text{K}^+$ ,  $\text{Rb}^+$ , or  $\text{NH}_4^+$  coming out of the protoplasm. It is interesting to note that, in spite of differences in behavior on entering the protoplasm, all three ions exhibit the same type of P D -time curve when coming out.

Four measurements with 0.01 molar  $\text{NH}_4\text{Cl}$  in sea water showed rather large changes in P D; in one case as much as 25 mv after 30 minutes. Similar results were obtained in three measurements with 0.001 molar  $\text{NH}_4\text{Cl}$  in sea water, where an increase of as much as 19 mv was observed. With both solutions, however, the rise in P D was gradual, and did not permit assigning even an approximate value to the initial change.

The measurements with 0.1 molar  $\text{NH}_4\text{Cl}$  in sea water are therefore the only ones which provide data suitable for computing the apparent relative mobility of  $\text{NH}_4^+$  in the X layer of *Valonia*. The same assumptions are made as in calculating the mobility of  $\text{Rb}^+$ . If we take as the initial change in P D the value, 68 mv, from the experiment

shown in Fig 4, the apparent mobility of  $\text{NH}_4^+$  is found to be 108 times as great as that of  $\text{Cl}^-$ . If we use 44 mv (the average of four discordant values) the mobility of  $\text{NH}_4^+$  is found to be 37 times as great as that of  $\text{Cl}^-$ . In any case, it seems clear that the apparent mobility of  $\text{NH}_4^+$  in the X layer is far greater than that of any of the other ions included in this study

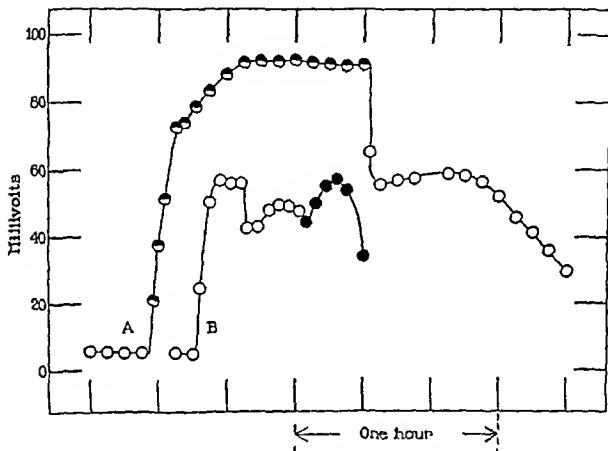


FIG 4 Time curves showing changes in P.D. across *Valonia* protoplasm produced by solutions containing  $\text{NH}_4\text{Cl}$ . Curve A shows the changes when natural sea water (open circles) is replaced by 0.1 molar  $\text{NH}_4\text{Cl}$  in sea water (half shaded circles) and when this solution in turn is replaced by natural sea water. Curve B shows the P.D.s with three different dilutions of  $\text{NH}_4\text{Cl}$  sea water: a 16.7 fold dilution (double circles), open centers; a fivefold dilution (double circles shaded centers); and a 3.3 fold dilution (shaded circles).  $\text{NH}_4\text{Cl}$  sea water contains 0.5 M  $\text{NH}_4\text{Cl}$ .

The high mobility of the  $\text{NH}_4^+$  ion suggests that the concentration effect with  $\text{NH}_4\text{Cl}$  sea water will be similar to the effects with  $\text{KCl}$  sea water and  $\text{RbCl}$  sea water. In particular, it is to be expected that the P.D. dilution curve will pass through a minimum.

That this is true is shown in a qualitative manner by the experiment



reported in the  $P D$ -time curve B, in Fig 4. It will be seen that the  $P D$  with the fivefold dilution of  $NH_4Cl$ -sea water is less than the  $P D$  with the 16.7-fold dilution which preceded it, and also less than the  $P D$  with the 3.3-fold dilution which followed it.

Such experiments, however, in which several dilutions of  $NH_4Cl$ -sea water are applied successively to the same cell, cannot be trusted to furnish quantitative data unless all the dilutions are greater than the critical value. Otherwise,  $NH_4^+$  ions entering from one solution are liable to cause changes at inner layers of the protoplasm and affect the value of the  $P D$  with later solutions.

A number of additional measurements with these three diluted  $NH_4Cl$ -sea waters failed to furnish more quantitative information. Since all three dilutions (with the possible exception of 16.7) are evidently less than the critical value,  $NH_4^+$  ions will enter the protoplasm and cause changes in  $P D$  at the inner layers. For quantitative comparisons, it is therefore necessary to determine the initial change in  $P D$  before  $NH_4^+$  ions have diffused through the  $X$  layer. Unfortunately, the shapes of the  $P D$ -time curves usually obtained with these solutions gave no clew to the magnitude of this initial change in  $P D$ .

#### DISCUSSION

The reason why the  $P D$ -time curves with sea waters containing  $RbCl$  or  $NH_4Cl$  do not rise sharply to well defined initial maxima can probably be traced to the greater speed with which these ions penetrate the protoplasm. If the curve is to show such a maximum, it is necessary that the entire<sup>10</sup> outer surface of the protoplasm shall be brought into equilibrium with the new solution before the  $P D$ 's at inner layers of the protoplasm have been affected by the penetration of ions from the new solution. If the penetration of  $NH_4^+$  (or  $Rb^+$ ) is too rapid, however, it may not be possible to meet this requirement since an appreciable time is needed to leach out the cell wall and bring the whole surface of the protoplasm in contact with the new solution. In an extreme case,  $NH_4^+$  ions might penetrate as far as the  $Y$  layer at one end of the cell before the  $NH_4Cl$ -sea water had reached the  $X$

<sup>10</sup> For a demonstration of the importance of applying the new solution to the entire outer surface of the cell, see Damon, E. B., *J. Gen. Physiol.*, 1932-33, 16, 376.

layer at the other end. The observed change in  $P D$  would then be the resultant of a very complex set of simultaneous changes, far too intricate to be interpreted.

If this explanation is correct, it should be possible to obtain  $P D$  time curves with well defined maxima and minima (like the curves with  $KCl$  sea water) by retarding the inward diffusion of  $NH_4^+$  through the  $X$  layer of the protoplasm. This can be accomplished by employing dilute solutions (below the critical dilution) where the entrance of  $NH_4^+$  is opposed, but not wholly prevented, by the potential gradient which arises from the outward diffusion of  $Na^+$  and  $Cl^-$  from the vacuole. Fig 5 shows the result of such an experiment, in which a threefold dilution of natural sea water was replaced by an equal dilution of a modified sea water containing 0.01 mole of  $NH_4Cl$  in a liter of the diluted solution. (Another such experiment, using fivefold dilutions, had exactly similar results.) It will be seen that the entrance of  $NH_4^+$  from the diluted sea water led to a  $P D$  time curve of precisely the same form as the curves resulting from the entrance of  $K^+$  from undiluted  $KCl$  rich sea waters. When the cell was returned to diluted natural sea water, the fluctuations in  $P D$  caused by  $NH_4^+$  coming out of the protoplasm were just the reverse of those caused by  $NH_4^+$  going in.

We may therefore conclude that sea waters with added  $KCl$ ,  $RbCl$ , and  $NH_4Cl$  would all give  $P D$ -time curves of essentially the same form if these curves could be measured under ideal conditions. In such ideal experiments, the solutions would be applied instantaneously to the entire outer surface of the protoplasm, and the measuring instrument would be rapid enough to follow accurately all the fluctuations in  $P D$ .

The greater speed with which  $Rb^+$  and  $NH_4^+$  enter the protoplasm is presumably the result of several contributing factors. Some of these will be discussed briefly.

For example, the concentration gradient in the protoplasm is doubtless greater for  $Rb^+$  than for  $K^+$ , since it may be expected that the protoplasm will normally contain a small concentration of  $K^+$ , but will not contain any perceptible amount of  $Rb^+$ . This somewhat steeper concentration gradient will be of greater importance when

the concentration of Rb in the external solution is small. The experiments with 0.1 molar RbCl in sea water furnish an example of the effect of small changes in the concentration gradient in the protoplasm. With cells which had been taken directly from ordinary sea water (and hence contained no  $\text{Rb}^+$ ) the p.d.-time curves showed, at best, only a badly defined point of inflection. With cells which had been exposed for a short time to a sea water containing 0.05 molar RbCl,

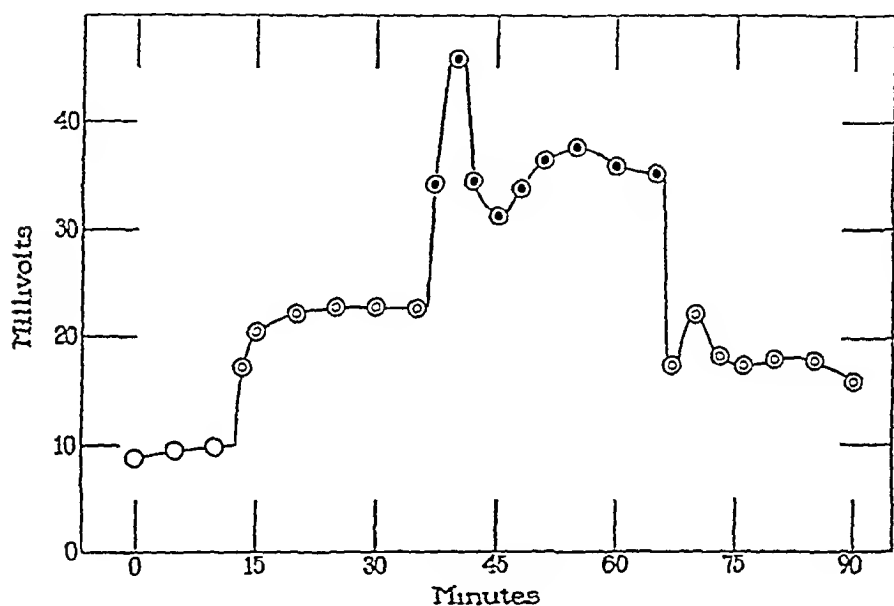


FIG. 5. p.d.-time curve showing the effect of substituting  $\text{NH}_4\text{Cl}$  for  $\text{NaCl}$  and  $\text{KCl}$  in diluted sea water. Open circles represent the p.d. in natural sea water, double circles with open centers, the p.d. in a threefold dilution of natural sea water, double circles with shaded centers, the p.d. in a threefold dilution of a modified sea water containing 0.01 mole of  $\text{NH}_4\text{Cl}$  in a liter of the diluted solution.

so that the protoplasm contained a little  $\text{Rb}^+$ , treatment with 0.1 molar RbCl in sea water caused the p.d. to rise rapidly and pass through a well defined maximum. This difference in behavior may reasonably be attributed to the slower inward diffusion of  $\text{Rb}^+$  in the second case, where the concentration gradient was less steep.

The speed with which  $\text{NH}_4^+$  penetrates the protoplasm might be explained, in part, by the hypothesis that uncharged  $\text{NH}_3$  molecules diffuse rapidly through the  $X$  layer, and on reaching the aqueous  $W$  layer react with water or some weak acid to form  $\text{NH}_4^+$  ions.

The concentration effect with higher dilutions of  $\text{NH}_4\text{Cl}$  sea water, however, must be considered as evidence against this hypothesis. With these dilute solutions, the potential gradient set up by the outward diffusion of  $\text{Na}^+$  and  $\text{Cl}^-$  from the vacuole opposes the inward diffusion of  $\text{NH}_4^+$  and other cations, but can have no effect on the diffusion of uncharged  $\text{NH}_3$  molecules. Since it is found that the influence of  $\text{NH}_4\text{Cl}$  on the P D is greatly diminished (and probably finally annulled) when the external solution is diluted, we must conclude that it is principally the  $\text{NH}_4^+$  ion, and not the  $\text{NH}_3$  molecule, which enters the protoplasm from these  $\text{NH}_4\text{Cl}$  rich solutions.

It is probable that differences in the partition coefficients for  $\text{KCl}$ ,  $\text{RbCl}$ , and  $\text{NH}_4\text{Cl}$  (between water and the  $X$  layer) have a very important effect on the rates at which  $\text{K}^+$ ,  $\text{Rh}^+$ , and  $\text{NH}_4^+$  enter the protoplasm. Since the values of these coefficients are unknown, it has been assumed for purposes of calculation that the coefficients are all equal. It must therefore be emphasized that the numerical values obtained from these calculations represent merely the *apparent* relative mobilities of the ions.

It has been pointed out elsewhere<sup>16</sup> that much higher dilutions of  $\text{KCl}$  sea water than of natural sea water can be applied to the *Valonia* cell without at once producing certain secondary changes which are probably a sign of injury. It is interesting that equally high dilutions of  $\text{RhCl}$  sea water or  $\text{NH}_4\text{Cl}$  sea water (which do not contain potassium) may be applied to *Valonia* for a short time without causing these secondary changes. That is, these ions which resemble  $\text{K}^+$  in their effect on the P D also exert the same protective action on *Valonia* protoplasm.

#### SUMMARY

In their influence on the P D across the protoplasm of *Valonia macrophysa*, Kütz,  $\text{Li}^+$  and  $\text{Cs}^+$  resemble  $\text{Na}^+$ , while  $\text{Rb}^+$  and  $\text{NH}_4^+$  resemble  $\text{K}^+$ . The apparent mobilities of the ions in the external surface layer of *Valonia* protoplasm increase in the order  $\text{Cs}^+$ ,  $\text{Na}^+$ ,  $\text{Li}^+ < \text{Cl}^- < \text{Rb}^+ < \text{K}^+ < \text{NH}_4^+$ .



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All the marine fish livers here examined contain a marked predominance of vitamin A<sub>1</sub>, but the sand flounder and herring, which possess this vitamin exclusively in the eye, contain in addition vitamin A<sub>2</sub> in the liver oils. Similarly the halibut liver, in which the presence

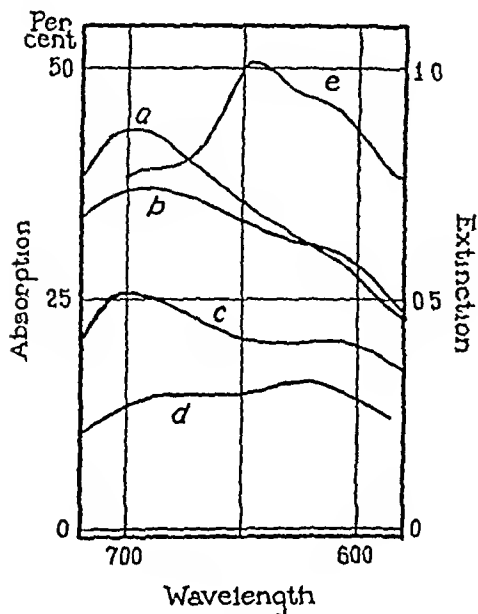


FIG 7

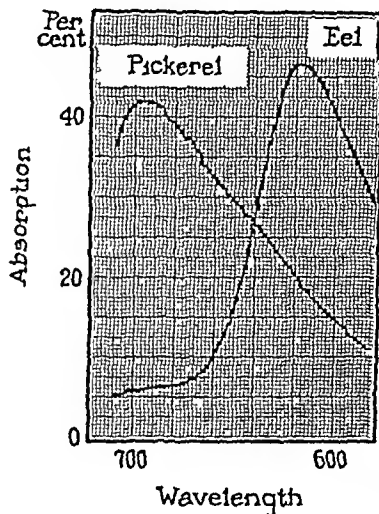


FIG 8

FIG 7 Combined pigmented epithelia and choroids of euryhaline fishes. Antimony chloride reactions with chloroform extracts from (a) seven chinook salmon, (b) seven rainbow trout, (c) seven brook trout, (d) seven eels, and (e) with a benzine extract from six white perch. All these tissues contain mixtures of vitamins A<sub>1</sub> and A<sub>2</sub>, and, with the possible exception of the white perch, predominantly the vitamin A ordinarily associated with the spawning environment. Ordinates for curve *c* are extinctions, for all the other curves percentages absorbed.

FIG 8 Antimony chloride reactions with liver oils from pickerel and eels caught in the same inland fresh-water pond. The pickerel liver contains vitamin A<sub>2</sub> alone, the eel about 99 per cent vitamin A<sub>1</sub>. The curves are original photoelectric recordings.

of a 690–695 mμ chromogen was first reported (Heilbron *et al*, 1931), contains about  $\frac{1}{10}$  as much vitamin A<sub>2</sub> as A<sub>1</sub>.

The fresh-water pickerel and calico bass livers contain vitamin A<sub>2</sub> exclusively, as do their eye tissues. The carp, however, which pos-

sesses only vitamin A in its eye, contains predominantly vitamin A<sub>1</sub> in its liver

The livers of all the anadromous fishes examined contain mixtures of both vitamins A. The proportions in the alewife and rainbow trout are like those in the eyes, while in the chinook salmon and brook trout these proportions are reversed

The catadromous eel contains almost exclusively vitamin A<sub>1</sub> in its liver. It is more "marine" in this respect than many of the permanently marine fishes, and this property is unaltered by years of fresh water existence. It is significant that the eel, pickerel, and calico bass liver data in Table II were derived from fishes all caught in the same inland fresh water pond, where they had shared the same environment and presumably largely the same food. Yet the proportions of vitamins A in the eel liver are almost diametrically the opposite of those in the permanently fresh water forms. This relation is demonstrated very strikingly in Fig 8. It is clear that the vitamin A configuration of the liver, like that of the eye, is determined by genetic and not by environmental factors

This conclusion is confirmed in an observation on chinook salmon livers. Those which yielded the data in Table II were obtained from yearling hatcheries fish which had never left fresh water. Similar extracts have been prepared from livers of adult fishes, caught at the mouth of the Columbia River at the beginning of their spawning migration. The proportion of vitamin A<sub>1</sub> to A<sub>2</sub> found in these mature fish was as 88 to 12, not seriously different from that in the fresh water parr

It may be concluded, therefore, that though the vitamin A pattern of the liver is not correlated as closely as that of the eye with the salinity type, it is equally independent of environmental fluctuation and is to an equal degree a racial characteristic<sup>6</sup>

<sup>6</sup> This conclusion should apply strictly only to what may be termed endogenous vitamin A, that is, vitamin A synthesized by the organism itself from carotenoid precursors. Lederer and Rathmann (1938) have shown that rats and frogs the livers of which normally contain little or no vitamin A<sub>2</sub> can accumulate this vitamin when fed fresh water fish liver concentrates. It is probable that by similar means the vitamin A configuration in the liver of any animal might be displaced in either direction

## DISCUSSION

The observations reported above permit a tentative statement of the significance of the vitamin A pattern of eye and liver oils in fishes. This is best introduced by a recapitulation of the argument dispersed throughout the foregoing pages.

The eye tissues of all marine fishes examined except the tautog contain exclusively vitamin A<sub>1</sub>, those of all the fresh-water fishes contain only vitamin A<sub>2</sub>, those of all euryhaline fishes, with the possible exception of the alewife, possess mixtures of both vitamins, and always predominantly that one which ordinarily is associated with the spawning environment. The liver oils in general share these relations imperfectly, usually they contain mixtures of both vitamins A, occasionally in reverse proportions to the eye tissues. These observations are summarized numerically in Table II.

The vitamin A configuration might be determined by the environment of the embryo, or of the mature fish, or it might be genetic in origin.

I The vitamin A pattern is not determined by the spawning environment, though highly correlated with it.

*a* Viviparous dogfishes possess vitamin A<sub>1</sub> alone, like many oviparous marine fishes.

*b* The eyes and livers of euryhaline fishes contain both vitamins, though each species spawns in either fresh water or in the sea.

*c* Tautog eye tissues contain predominantly vitamin A<sub>2</sub>, though this fish spawns in the sea.

II The vitamin A pattern is not determined by the salinity of the adult environment.

*a* Salmonids which have never left fresh water still possess both vitamins A. Their proportions in chinook salmon livers are essentially the same in yearling fresh-water fish and in mature animals just in from the ocean.

*b* The eel retains a great predominance of vitamin A<sub>1</sub> after years of existence in fresh water, the alewife a predominance of A<sub>2</sub> after years in the sea.

*c* The eye tissues of mature marine tautogs contain primarily vitamin A<sub>2</sub>.

III The vitamin A pattern is not determined by nutrition.

a The vegetarian carp possesses the same pattern in its eye tissues as the carnivorous pickerel and calico bass

b Conversely, eels caught in the same pond as pickerel and calico bass, and presumably sharing the same food, possess the reverse type of vitamin A pattern

c Eels caught in salt marshes at the beginning of their spawning migration seaward possess empty digestive tracts, having apparently ceased to feed. After being kept subsequently in fresh water without food for an additional 4 months, they possess the same eye and liver vitamin A patterns as eels freshly caught in an inland pond

I conclude that the vitamin A configurations of both eye and liver are determined by genetic factors. Presumably these operate through specific enzyme systems which govern the conversion of dietary carotenoids, ultimately of plant origin, into the vitamins A.

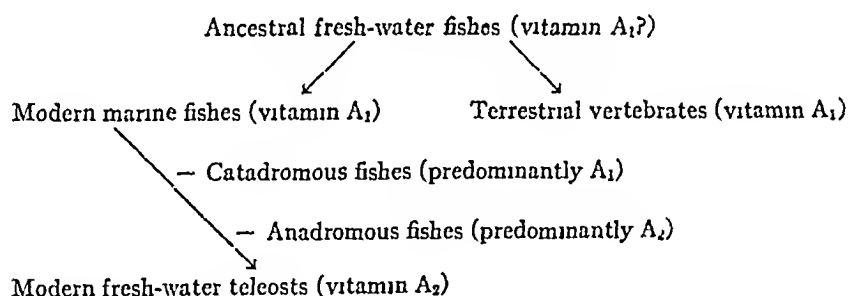
The conditions of steno- and euryhalinity and the choice of spawning environment are also inherited. We have to deal therefore with the correlation of two types of genetic character rather than with some labile functional relation. The significance of this correlation is to be sought in the phylogeny rather than in the physiology of the fishes.

Considerable anatomical and paleontological evidence indicates that all fishes originated in fresh water (Smith, 1932). The ancestors of modern marine forms appear to have migrated to the sea principally in late Paleozoic and early Mesozoic times (Romer and Grove, 1935). The modern fresh water fishes, however, may be divided into two groups: (1) those like the lung fishes which probably have continuously occupied fresh water environments, none of these has yet been included in the present research, and (2) the modern fresh water teleosts, which, it is generally agreed, have re-entered fresh water from the sea. In this sense the fresh water teleosts are secondarily derived from the marine teleosts, and so represent the most recent general evolutionary development among the fishes.<sup>7</sup>

The connection between this development and the distribution of vitamins A may be formulated tentatively as follows. The ancestral fresh water fishes probably possessed vitamin A<sub>1</sub> alone, and communicated this to both their marine and terrestrial descendants. This is

<sup>7</sup> I am greatly indebted to Professor A. S. Romer of Harvard University for a critical discussion of the phylogeny of the fishes.

therefore the vitamin A almost universal among the vertebrates. It may still be retained by the continuously fresh-water line of lung fishes. In the modern teleosts two evolutionary trends emerge: re-migration to fresh water, and assumption of vitamin A<sub>2</sub> metabolism. Considering only the eye tissues, the stenohaline marine fishes, with few exceptions, have retained exclusively vitamin A<sub>1</sub>. The stenohaline fresh-water forms have negotiated completely both migration and the transfer to vitamin A<sub>2</sub>. The euryhaline fishes, which may be regarded as transitional in the migratory sense, possess intermediate mixtures of both vitamins A. If the anadromous alewife, white perch, and salmonids may be considered to be further advanced in their evolutionary migration to fresh water than the catadromous eel, even these distinctions are reflected faithfully in the vitamin A patterns (see diagram). The correlation between both these evolutionary developments is well nigh complete. Yet apparently one may occur without the other, since the tautog has developed a predominantly vitamin A<sub>2</sub> visual system without to our knowledge ever having left the sea.



This situation offers a comparatively recent parallel to the alteration in muscle phosphagens associated with the origin of the vertebrates. Almost all invertebrate muscles possess arginine phosphate alone, an echinoderm (*Strongylocentrotus*) and a primitive chordate (*Balanoglossus*), both believed by some paleontologists to represent intermediate types in the progression from invertebrates to vertebrates, possess mixtures of arginine and creatine phosphates, and *Amphioxus* and the vertebrates possess creatine phosphate alone (Meyerhof, 1930<sup>8</sup>, Needham, Needham, Baldwin, and Yudkin, 1932).

Perhaps also comparable with the vitamin A situation is the synthesis in certain fishes of trimethyl amine oxide Hoppe Seyler and Schmidt (1927) have found this product in all marine fishes examined, but not a trace of it in fresh water forms It is significant, however, that among the latter the authors include the Atlantic salmon and the European fresh water eel This property therefore does not appear to be intermediate in the euryhaline fishes, and may, as the authors suggest, be a direct environmental response

There is no evidence that any of these biochemical changes constitutes an evolutionary advantage The substitution of creatine for arginine, trimethyl amine oxide for other nitrogenous end products, or vitamin A for A<sub>1</sub> entails no obvious increase in fitness On the other hand the change from sea to fresh water or the assumption of a notochord are not similarly indifferent It is probably because they are genetically bound to such highly selective changes that these relatively neutral biochemical developments are perpetuated

#### SUMMARY

The distribution of vitamins A<sub>1</sub> and A<sub>2</sub> has been determined in the eye tissues and livers of a number of fishes The vitamins were differentiated by means of the antimony chloride reaction, which yields with A<sub>1</sub> a band at 615-620 m $\mu$  and with A<sub>2</sub> a band at about 696 m $\mu$  In the retina the presence of vitamin A<sub>1</sub> is diagnostic of the operation of a rhodopsin, and vitamin A<sub>2</sub> of a porphyropsin cycle

The eye tissues of all permanently marine fishes examined, except the tautog, contain vitamin A<sub>1</sub> alone Those of all permanently fresh water fishes possess only vitamin A Those of all euryhaline (potentially migratory) fishes, except possibly the alewife, contain mixtures of both vitamins A, and always predominantly that one which ordinarily is associated with the environment in which the fish is spawned

These correlations extend in part to the liver oils, but most livers contain mixtures of both vitamins A, and occasionally in proportions the reverse of those in the eye tissues

The vitamin A configuration does not depend upon environmental circumstances, but is determined genetically The transfer from vitamin A<sub>1</sub> to A<sub>2</sub> metabolism appears associated phylogenetically with migration of marine teleosts into fresh water

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# CHANGES OF APPARENT IONIC MOBILITIES IN PROTOPLASM

## IV INFLUENCE OF GUAIACOL ON THE EFFECTS OF SODIUM AND POTASSIUM IN NITELLA

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Guaiacol not only changes the behavior of *Valonia*<sup>1</sup> and of *Halimys*,<sup>2</sup> but, as this paper demonstrates, is likewise potent in *Nitella*. The results show differences as well as similarities in the three species and a comparison is instructive.

In normal cells of *Nitella* the effect of  $K^+$  on the P.D. is so much greater than that of  $Na^+$  that normal cells are able to distinguish between  $K^+$  and  $Na^+$  in somewhat the fashion of a potassium electrode.

This ability is greatly lessened by guaiacol (hereafter called HG for convenience) because it increases the effect of  $Na^+$  on P.D. but not that of  $K^+$ . As a result  $Na^+$  acts more like  $K^+$ .

This ability is likewise lessened by leaching in distilled water which removes an organic substance (or group of substances) called for convenience<sup>3</sup>  $R_p$ . In this case the effect of  $K^+$  is lessened but not that<sup>4</sup> of  $Na^+$ .

In both cases the effects of  $K^+$  and  $Na^+$  are made to approach each other and their action on P.D. becomes more nearly identical.

The effect of HG shown in Fig. 1 is fairly typical except that recovery is somewhat quicker than usual.<sup>5</sup>

<sup>1</sup> Osterhout, W J V, *J Gen Physiol*, 1936-37, 20, 13

<sup>2</sup> Osterhout, W J V, *J Gen Physiol*, 1937-38, 21, 707

<sup>3</sup> Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1933-34, 17, 105 Hill, S E, and Osterhout, W J V, 1937-38, 21, 541

<sup>4</sup> Osterhout, W J V, and Hill, S E, *Proc Nat Acad Sc*, 1938, 24, 427

<sup>5</sup> The experiments were performed on *Nitella flexilis*, Ag., using the technique employed in former papers (cf. Hill, S E, and Osterhout, W J V, *J Gen Physiol*, 1937-38, 21, 541). Temperature 20 to 25 C. The cells were freed from neighbor

At the start the P D is positive<sup>6</sup> to the extent of 100 mv. When 0.01 M NaCl + 0.025 M HG is applied the P D begins to fall off so that the curve rises, suggesting that in the presence of HG the behavior of  $\text{Na}^+$  becomes more like that of  $\text{K}^+$  which has a strongly negativating effect.<sup>7</sup>

This might be due to an increase in  $u_{\text{Na}}$ , the mobility of  $\text{Na}^+$  in the outer protoplasmic surface,  $X$  (see Fig. 2), or to an increase in its partition coefficient  $S_{\text{Na}}$  ( $S_{\text{Na}}$  = concentration of  $\text{Na}^+$  in  $X$  — concentration of  $\text{Na}^+$  in external solution).

Presumably we should then have the following situation in  $X$

$X$	
Region in equilibrium with 0.001 M NaCl	Region in equilibrium with 0.001 M NaCl + HG
$P_1$	

We might assume that the falling off in P D caused by guaiacol is due to the inwardly directed (negative) diffusion potential<sup>8</sup> at  $P_1$  caused by the action of guaiacol in raising the value of  $u_{\text{Na}}$  or  $S_{\text{Na}}$ . Such alterations do not seem improbable since HG changes mobilities in *Valonia*<sup>1</sup> and in *Halicystis*<sup>2</sup> in *Nilrella* partition coefficients can be changed by various means, e.g. by distilled water,<sup>4</sup> by action currents,<sup>9</sup> and by calcium.<sup>10</sup>

ing cells and kept for 30 days or more at  $15 \pm 1^\circ\text{C}$  in Solution A (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87). These cells belonged to Lot B (cf. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sci.*, 1938, 24, 312, cf. also Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 541; Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938-39, 22, 139). They were kept at room temperature for about 2 hours before the experiment was performed.

There was no sign of injury in these experiments, the concentration of HG was in all cases 0.015 to 0.025 M (which gave similar effects), the use of 0.03 M HG was avoided as it may be toxic in longer experiments.

It may be added that the cells are quite variable and in order to avoid confusion the presentation has adhered in general to the more typical behavior.

<sup>6</sup> I.e., the positive current tends to flow across the protoplasm from the sap to the external solution.

<sup>7</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715; Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 541.

<sup>8</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715.

<sup>9</sup> Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 541.

<sup>10</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938-39, 22, 139.

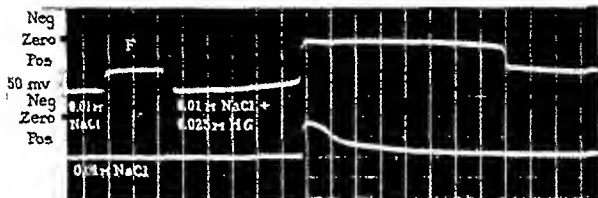


FIG 1 The effect of HC as shown by a photographic record of changes at C and D (Fig 2) at the start the spot F was in contact with 0.01 M KCl

At the start the p.d. at C (upper string) was 100 mv positive in 0.01 M NaCl. When the solution was withdrawn from C the curve jumped to F, the free grid potential of the amplifier. When 0.01 M NaCl + 0.025 M HG was applied at C the curve dropped back and then rose slowly. This slow rise was followed by an action current with delayed recovery; the recovery curve descends suddenly giving a square topped action current.

The action current was propagated to D (lower string) here the action current has a normal appearance. D was in contact with 0.01 M NaCl.

Heavy time marks 5 seconds apart. Temperature 26°C.

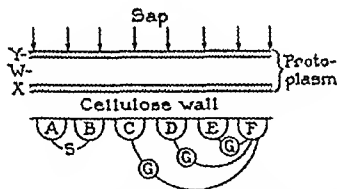


FIG 2 Diagram to show the arrangement of leads and the supposed structure of the protoplasm which is assumed to consist of an aqueous layer II, an outer non aqueous layer I, and an inner non aqueous layer J.

The arrows show the outwardly directed (positive) p.d. whose seat is supposed to be chiefly at I when the cell is in pond water. Hence the p.d. at I is regarded as negligible and is not shown. But under some conditions the p.d. at I may become important.

Each lead is connected to a separate amplifier and to one string of the 3 string Enthoven galvanometer.

As is evident from previous papers,<sup>7, 11</sup> we can tell whether  $u_{Na}$  is altered if we measure the concentration effect. An increase of  $u_{Na}$  would increase the concentration effect, but an increase of  $S_{Na}$  would not do so. The concentration effect was measured, as shown in Fig. 3. Before applying HG the substitution of 0.01 M for 0.001 M NaCl (and *vice versa*) gives  $23.2 \pm 0.9$  mv (22 observations). From this we calculate, by methods described in a previous paper,<sup>8</sup> the value of  $u_{Na}$  as 2.33. A similar measurement, made after the application of HG and the resulting action current, is illustrated in Fig. 3. The

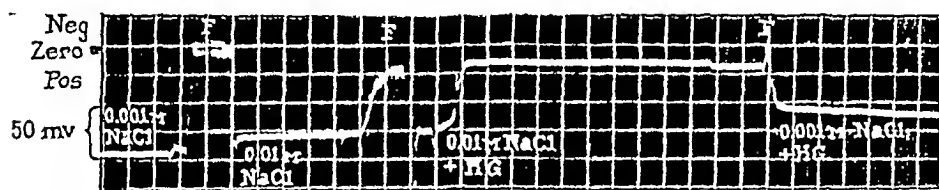


FIG. 3. Action of HG on the concentration effect of NaCl as shown by a photographic record of changes in PD at C (Fig. 2) which at the start was in contact with 0.001 M NaCl (*F* was in contact with 0.01 M KCl).

When 0.01 M NaCl was applied the curve rose 18 mv. When 0.01 M NaCl + 0.025 M HG was applied the curve rose slowly and an action current occurred. When 0.001 M NaCl + 0.025 M HG was applied the curve fell 48 mv.

Heavy time marks 5 seconds apart. Temperature 22°C.

Regarding *F* see Fig. 1.

average of 39 observations<sup>12</sup> is  $44.0 \pm 0.7$  mv. From this we get  $u_{Na} = 7.30$ , a value considerably higher than before HG was applied. The change from  $u_{Na} = 2.33$  to  $u_{Na} = 7.30$  makes the PD more negative. The amount can be calculated as follows:

Since in the presence of HG the behavior of  $Na^+$  is somewhat like that of  $K^+$  we may for purposes of calculation regard it as playing the role of  $K^+$ . Hence replacing 0.01 M NaCl with  $u_{Na} = 2.33$  by 0.01 M NaCl with  $u_{Na} = 7.30$  might be regarded as equivalent to replacing 0.01 M NaCl by 0.01 M KCl. If in the latter

<sup>11</sup> Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312.

<sup>12</sup> This does not include certain low values obtained directly after the spike on passing from 0.001 M to 0.01 M NaCl, see Fig. 4. It includes values obtained on passing from 0.001 M to 0.01 M NaCl and *vice versa* taken later on in the course of the action current, see p. 422.

case the concentration of  $\text{Cl}^-$  in  $\lambda$  remains constant we have according to Henderson's equation (at 20 C)

$$\text{Change of } P.D. = 58 \log \frac{\mu_K + r_{\text{Cl}}}{\mu_{\text{Na}} + r_{\text{Cl}}}$$

Substituting the numerical values<sup>13</sup> of  $\mu_{\text{Na}}$  we have

$$\text{Change of } P.D. = 58 \log \frac{7.30 + 1}{2.33 + 1} = 23 \text{ mv}$$

Evidently the change from  $\mu_{\text{Na}} = 2.33$  to  $\mu_{\text{Na}} = 7.30$  can make the  $P.D.$  23 mv more negative. As this is not enough to account in all cases for the total upward movement of the curve during its slowly rising phase it seems probable that  $S_{\text{Na}}$  has also increased. This is shown to be the case by measurements of the potassium effect (i.e. the change in  $P.D.$  observed on substituting  $\text{KCl}$  for  $\text{NaCl}$ ) which will be discussed later (p. 426).

We might therefore attribute the upward movement of the curve in its slowly rising phase in Fig. 1 to the increase<sup>14</sup> in  $\mu_{\text{Na}}$  and  $S_{\text{Na}}$ . But we find that in some cases curves somewhat resembling the upper curve in Fig. 1 are obtained on applying 0.02 M HG in tap water. The cause of this is not clear; it might be regarded as an effect of HG on  $Y$  (Fig. 2) but it would then be necessary to assume a very rapid penetration of HG in such cases.

When the  $P.D.$  has fallen off sufficiently<sup>15</sup> in the slowly rising phase of the curve an action current<sup>16</sup> occurs. This is probably due, as explained in a previous paper,<sup>9</sup> to a discharge from a neighboring region and an exit of substances from the sap in that region. If these substances diffuse along  $W'$  (Fig. 2) to the spot in contact with guaiacol they may cause an action current at that spot.<sup>17</sup>

<sup>13</sup> As in previous papers we put  $r_{\text{Cl}} = 1$  cf. footnote 8.

<sup>14</sup> This increase in  $\mu_{\text{Na}}$  and  $S_{\text{Na}}$  also occurs when the cell is in contact with 0.001 M or 0.01 M  $\text{KCl}$  when the curve does not rise (Fig. 5).

<sup>15</sup> When 0.001 M  $\text{NaCl} + \text{HG}$  does not depress the  $P.D.$  sufficiently to cause an action current subsequent application of 0.01 M  $\text{NaCl} + \text{HG}$  will do so. Or electrical stimulation may be applied.

<sup>16</sup> This is frequently propagated along the cell (Fig. 1).

<sup>17</sup> As would be expected an action current can be produced at any time during the slowly rising phase of the curve (Fig. 1) by electrical stimulation. This resembles the action current which occurs without such electrical stimulation.

The delay in recovery may be due to the action of HG upon  $Y$  (Fig 2) The long delay in recovery and the sudden descent of the curve in recovery, giving "square topped" action curves, recalls the situation in cells treated with guanidine<sup>18</sup>

We suppose<sup>19</sup> that the action current involves a loss of  $PD$  at  $Y$  (Fig 2) If  $Y$  recovers its normal  $PD$  we expect in the present case only partial recovery since in the presence of HG NaCl acts upon  $X$  (Fig 2) somewhat like KCl (because either  $\mu_{Na}$  or  $S_{Na}$  or both have increased) and thus produces an inwardly directed (negative)  $PD$  which lessens the net total outwardly directed (positive)  $PD$  Hence, in Fig 1, the level of the curve after recovery is higher than before HG was applied It would be still higher but for the fact that after recovery  $\mu_{Na}$  and  $S_{Na}$  fall off<sup>20</sup> the cause of this is not clear

It may be added that during the action curve seen in Fig 1 the stimulated region no longer appears to act as a condenser when tested in a Wheatstone bridge as described by Blinks<sup>21</sup> This applies also to the action curve in 0.001 M KCl seen in Fig 6 In both these cases we may suppose that  $I^-$  (Fig 2) has lost its  $PD$  and a great part of its resistance

An interesting result is seen in Fig 4 where 0.001 M NaCl is replaced by 0.001 M NaCl + HG After a slow rise in the curve an action current occurs and 0.001 M NaCl + HG is then replaced by 0.01 M NaCl + HG and the curve quickly rises 32 mv On the basis of what has already been said regarding Fig 3 we should expect a rise of about 44 mv When the solution is changed back again to 0.001 M NaCl + HG the curve does not go back to the former level but drops much lower the total drop is 44 mv which is about what would be expected according to the discussion of Fig 3 (p 420)<sup>22</sup>

<sup>18</sup> Osterhout, W. J. V., and Hill, S. E., Some ways to control bioelectrical behavior, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1936, 4, 43. See also, Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 91.

<sup>19</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, 18, 215.

<sup>20</sup> This is shown by measurements of the concentration effect of NaCl and of the potassium effect (on the assumption that  $S_K$  and  $\mu_K$  remain constant as stated on p 424).

<sup>21</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 495, 1936-37, 20, 229. See also Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, 1938-39, 22, 37.

<sup>22</sup> This behavior of 0.01 M NaCl is not seen when it is applied soon after the spike

This can be accounted for if we assume<sup>23</sup> that when 0.01 M NaCl + HG is applied just after the spike of the action current some of it penetrates  $\lambda$  (Fig 2). This seems probable because the experiments of Blinks<sup>21</sup> and of Cole and Curtis<sup>21</sup> show that just after the spike of the action current the protoplasm becomes very permeable. Evidently the NaCl which penetrates sets up an outwardly directed (positive) P D against  $X$  opposing the inwardly directed P D due to the 0.01 M NaCl outside and in consequence we find less than the expected effect. After this, however, each change of concentration in either direction produces its full effect (i.e. the increased concentration effect of 45 to 50 mv) and the only effect of the NaCl which has penetrated to the region just inside  $\lambda$  is permanently to lower the level of the curve.<sup>25</sup>

In striking contrast to all that has been said about  $\text{Na}^+$  is the behavior of  $\text{K}^+$ , as seen in Fig 5. Here HG has practically no effect.<sup>27</sup> When the cell is stimulated electrically we get an action curve (Fig 6). The delay in recovery may be due to the action of HG upon  $Y$  (Fig 2). In the absence of HG electrical stimulation in 0.001 M KCl produces a normal action curve with recovery in about 20 seconds.<sup>8</sup>

of an action current produced by electrical stimulation when the cell is in contact with 0.001 M KCl + 0.02 M HG. Apparently the protoplasmic surface is then in a different state (see p. 425).

<sup>23</sup> If this assumption is not made we must suppose that the low concentration effect just after the spike of the action current means a value for  $u_{\text{Na}}$  about equal to the normal (i.e. about 2.33). The slow rise in the curve preceding the spike in the action current would then be attributed to a rise in  $S_{\text{Na}}$  rather than in  $u_{\text{Na}}$ . The increase in the concentration effect observed at the second application of 0.01 M NaCl after the spike might be attributed to a sudden rise in  $u_{\text{Na}}$  due to substances moving from the sap to  $\lambda$  (cf. footnote 9).

<sup>24</sup> Such values are included in the average of 44 mv given on p. 420.

<sup>25</sup> In some cases the curve after the spike of the action current drifts slowly downward when the cell is in contact with 0.001 M NaCl or 0.01 M NaCl; this may indicate penetration.

<sup>26</sup> A similar result is obtained in 0.01 M KCl.

<sup>27</sup> In a few cases the curve rises somewhat when HG is applied and an occasional cell is found in which  $\text{K}^+$  acts very much like  $\text{Na}^+$ . In some cases the curve falls somewhat under the influence of HG and rises somewhat above its original level when HG is removed.

<sup>28</sup> Osterhout W J V, *J Gen Physiol* 1934-35 18, 215.



If  $u_k$  or  $S_k$  were changed by HG the level of the curve in Fig 5 would change. Since there is little change we may conclude that  $u_k$  and  $S_k$  remain almost constant. This is confirmed for  $u_k$  by the following measurements

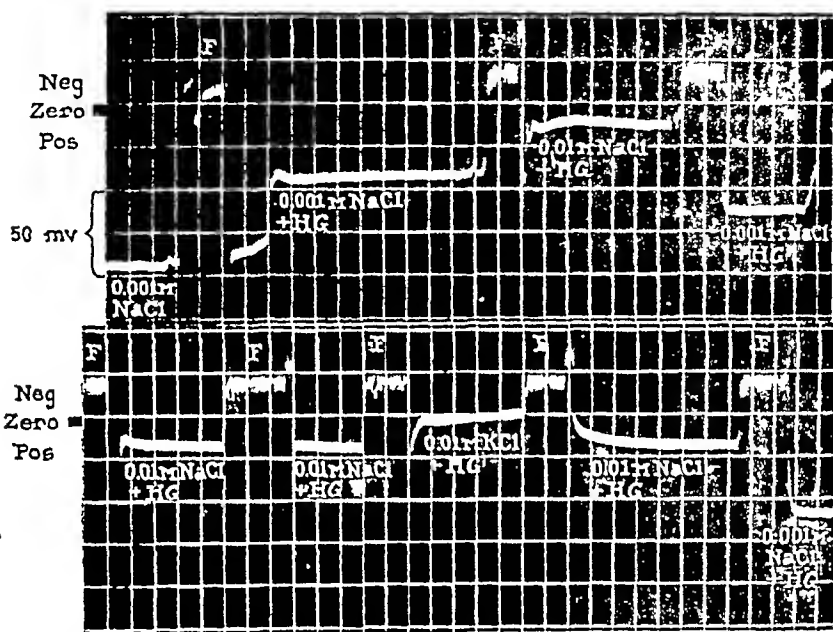


FIG 4 Action of HG on the concentration effect of NaCl and on the potassium effect as shown by a photographic record of changes of p.d. at D (Fig 2). The spot F was in contact with 0.01 M KCl.

At the start the p.d. was 94 mv positive in 0.001 M NaCl. When 0.001 M NaCl + HG was applied the curve rose slowly; this was followed by an action current. When 0.01 M NaCl + HG was applied the curve rose 32 mv. When 0.001 M NaCl + HG was applied the curve fell 50 mv. When 0.01 M NaCl + HG was applied the curve rose 45 mv (this solution was removed and again applied). When 0.01 M KCl + HG was applied the curve rose 18 mv and returned to the previous level when 0.01 M NaCl + HG was applied (potassium effect).

The concentration of HG was 0.025 M in all cases. Heavy time marks 5 seconds apart. Temperature 23°C.

Regarding F see Fig 1.

Before HG is applied the potassium concentration effect (change of 0.001 M to 0.01 M KCl<sup>29</sup> and *vice versa*) is  $48.5 \pm 0.7$  (33 observations).

<sup>29</sup> Large values due to action currents are rejected. Cf footnote 9.

during exposure to HG it is  $44.2 \pm 0.3$  (41 observations) before and  $45.8 \pm 0.6$  (44 observations) after electrical stimulation. This indicates that little or no change in  $u_K$  is produced by HG. Taking as a general average 46.1 we get<sup>8</sup>  $u_K = 8.76$ .

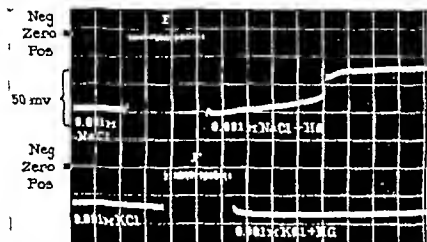


FIG 5 Contrasting effects of HG in the presence of NaCl and KCl as shown by a photographic record showing behavior of PD at C and D (Fig 2). At the start C was in contact with 0.001 M NaCl, D with 0.001 M KCl and F with 0.01 M KCl.

When 0.001 M NaCl at C (upper string) was changed to 0.001 M NaCl + 0.02 M HG there was a slow rise of the curve followed by an action current. This did not happen when 0.001 M KCl at D (lower string) was replaced by 0.001 M KCl + 0.02 M HG.

Heavy time marks 5 seconds apart. Temperature 23°C.

Regarding F see Fig 1.

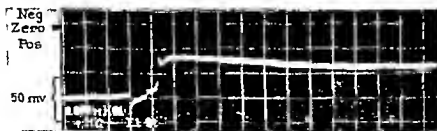


FIG 6 Effect of electrical stimulation as shown by a photographic record of changes of PD at C (Fig 2). C was in contact with 0.001 M KCl + 0.02 M HG. F was in contact with 0.01 M KCl.

When electrical stimulation was applied at S (Fig 2) an action current occurred at C (the preliminary part of the action curve is due to leakage). Recovery was delayed.

Heavy time marks 5 seconds apart. Temperature 23°C.

Regarding F see Fig 1.